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1. **The Double Patenting Rejection Should Be Withdrawn**

All pending claims are rejected under the judicially created doctrine of double patenting over claims 36-102 of U.S. application no. 09/113,399 ("the '399 application"). The Examiner contends that, although the claims pending in the present application and those pending in the '399 application are not identical, "they are not patentably distinct from each other because the claims of the instant application are an obvious variation of the claims of the '399 application." The Examiner continues to state that

> [t]he instant claims are broadly drawn to a method for altering cell fate comprising altering Notch pathway function in the cell by contacting the cell with a modulator of Notch pathway function. Said modulators also alter the function of a gene pathway that is not Notch. The claims of the '399 application are drawn to a method for the modulation of a signal transduction pathway by modulating Notch function (*i.e.*, Notch pathway) with an agonist such that a signal pathway that is not Notch is affected. Once of ordinary skill in the art would have recognized that the method involved in the alteration of cell fate in the instant application and the method for modulating a signal transduction pathway in the '399 application are the same. Therefore, the instant invention would have been obvious to one or ordinary skill in the art."

Applicants respectfully disagree. A finding of obviousness requires a determination of the scope and content of the prior art, the differences between the invention and the prior art, the level of ordinary skill in the art, and whether the differences are such that the subject matter as a whole would have been obvious to one of ordinary skill in the art at the time the invention was made. *Graham v. Deere*, 383 U.S. 1 (1966). The prior art must suggest the invention, and provide one of ordinary skill in the art with a reasonable expectation that the suggestion would work. *In re O'Farrell*, 853 F.2d 894, 903 (Fed. Cir. 1988). Applicants submit that the Examiner has not met her burden of establishing a *prima facie* case of obviousness of the presently pending claims over the claims of the '399 application.

First, Applicants respectfully submit the Examiner is mischaracterizing the presently claimed invention. The Examiner states that the present claims are "broadly" drawn to modulating cell fate by modulating Notch pathway activity, which "also alters the function of a gene pathway that is not Notch." However, the presently claimed invention is not merely

directed to a method of modulating cell fate by modulating Notch pathway activity, but to a method of altering cell fate by independently and concurrently altering Notch pathway function and the function of a cell fate control gene pathway. In contrast, the claims of the '399 application are directed to methods of modulation of a function of a signal transduction pathway by agonizing Notch function. The claims of the '399 application do not suggest, nor does they provide one of skill in the art with the motivation, to alter cell fate by independently and concurrently modulating Notch pathway function and cell fate control gene pathway function. Accordingly, the presently pending claims are not obvious over the claims of the '399 application.

In view of the foregoing, Applicants submit that the double patenting rejection has been obviated and should be withdrawn.

2. The Rejections Under 35 U.S.C. § 112, First Paragraph For Lack of Enablement, Should Be Withdrawn

Claims 1-5, 7-10, 13-15, 18-21, 24-34, 50-52, 63-66 and 69-83 are rejected under 35 U.S.C. § 112, first paragraph, allegedly because the specification does not enable one of skill in the art to practice the claimed methods. In particular, the Examiner characterizes the presently claimed invention as:

> broadly drawn to a method for altering cell fate comprising altering notch pathway function in the cell by contacting the cell with a modulator of Notch pathway function. Said modulator also alters the function of a gene pathway that is not Notch.

In view of the foregoing characterization of the present claims, the Examiner states that the present disclosure does not enable the claimed invention, as "the modulation of cell fate by altering Notch pathway functions is unpredictable with regard to achieving specificity in the inhibition of a signal transduction pathway."

Applicants respectfully assert that the Examiner's rejections under 35 U.S.C. § 112, first paragraph, for lack of enablement are based on a mischaracterization of the presently claimed invention. Applicants further submit that, for the reasons discussed below and according to the applicable case law, the instant specification does, indeed, fully enable one of skill in the art to practice the claimed invention.

THE LEGAL STANDARD

The test for enablement is whether one reasonably skilled in the art could make or use the invention, without undue experimentation, from the disclosure in the patent specification coupled with information known in the art at the time the patent application was filed. *U.S. v. Telectronics Inc.*, 857 F.2d 778, 8 U.S.P.Q.2d 1217 (Fed. Cir. 1988). In fact, well known subject matter is preferably omitted. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384 (Fed. Cir. 1986) ("a patent need not teach, and preferably omits, what is well know in the art."). Further, one skilled in the art is presumed to use the information available to him in attempting to make or use the claimed invention. *See Northern Telecom, Inc. v. Datapoint Corp.*, 908 F.2d 931, 941 (Fed. Cir. 1990) ("A decision on the issue of enablement requires determination of whether a person skilled in the pertinent art, using the knowledge available to such a person and the disclosure in the patent document, could make and use the invention without undue experimentation."). These enablement rules preclude the need for the patent applicant to "set forth every minute detail regarding the invention." *Phillips Petroleum Co. v. United States Steel Corp.*, 673 F. Supp. 1278, 1291 (D. Del. 1991); *see also DeGeorge v. Bernier*, 768 F.2d 1318, 1323 (Fed. Cir. 1985).

Undue experimentation is experimentation that would require a level of ingenuity beyond what is expected from one of ordinary skill in the field. *Fields v. Conover,* 170 U.S.P.Q. 276, 279 (C.C.P.A. 1971). The factors that can be considered in determining whether an amount of experimentation is undue have been listed in *In re Wands,* 8 U.S.P.Q.2d 1400, 1404 (Fed. Cir. 1988). Among these factors are: the amount of effort involved, the guidance provided by the specification, the presence of working examples, the amount of pertinent literature and the level of skill in the art. The test for undue experimentation is not merely quantitative, since a considerable amount of experimentation is permissible, so long as it is merely routine. *Id.*

Further, while the predictability of the art can be considered in determining whether an amount of experimentation is undue, mere unpredictability of the result of an experiment is not a consideration. Indeed, the Court of Custom and Patent Appeals has specifically cautioned that the unpredictability of the result of an experiment is not a basis to

conclude that the amount of experimentation is undue in *In re Angstadt,* 190 U.S.P.Q. 214 (C.C.P.A. 1976):

> [If to fulfill the requirements of 112, first paragraph, an applicant's] disclosure must provide guidance which will enable one skilled in the art to determine, with reasonable certainty before performing the reaction whether the claimed product will be obtained, . . . then all "experimentation" is "undue" since the term "experimentation" implies that the success of the particular activity is uncertain. Such a proposition is contrary to the basic policy of the Patent Act.

Id. at 219 (emphasis in the original).

THE INSTANT SPECIFICATION FULLY ENABLES THE PRESENTLY CLAIMED INVENTION

Contrary to the Examiner's contention that presently claimed invention is directed to "a method for altering cell fate comprising altering Notch pathway function in the cell by contacting the cell with a modulator of Notch pathway function [that] also alters the function of a gene pathway that is not Notch," the present invention is directed to methods of altering cell fate by concurrently and directly manipulating Notch pathway function (*e.g.*, by contacting the cell with a Notch agonist or antagonist, as described in Sections 5.3 and 5.4 of the specification) and the function of a cell fate control gene pathway (*e.g.*, by contacting the cell with an agonist or antagonist of the cell fate control gene, as described in Sections 5.6 and 5.7 of the specification). Step (b) of claim 1 clearly requires a modulator of a cell fate control gene pathway that is not a Notch agonist or antagonist, but rather one that is used in conjunction with the Notch agonist or antagonist.

In connection with this rejection, the Examiner states that "the specification does not teach how to specifically modulate Notch or any signal transduction pathway," given that there are cell context-dependent differences in the Notch pathway among the cell types covered by the breadth of the claims. Applicants submit that one of skill in the art can readily modulate (agonize or antagonize) Notch or cell fate control gene pathway activity a cell of interest using the teachings in the specification together with, if necessary, readily available information. In particular, the specification teaches a large number of ways to modulate Notch pathway activity in cells, most of which are applicable to all cell types. For example, and as discussed in the specification at page 36, lines 1-11, the ankyrin repeats of

in vitro fine
in vivo as a problem

the intracellular region of Notch protein are both necessary and sufficient for downstream signaling of the Notch receptor. A cell expressing a truncated form of Notch containing the ankyrin repeats will agonize the Notch pathway in *Drosophila*, and is expected to do the same in various cell types, regardless of the cell type of the cell, particularly as it is the region of Notch containing the ankyrin repeats that is the most highly conserved among species. Further, one of skill in the art would recognize that the Notch pathway would be antagonized in various cell types by using a Notch antisense nucleic acid, for example as described in the specification at pages 40-42 of the specification. Similarly, one can modulate the activity of a cell fate control gene pathway independently of cell type. For example, most cell fate control gene pathways can be agonized in a cell simply expressing the corresponding cell fate control gene in the cell or contacting the cell with the encoded protein (*e.g.*, as described in the specification at page 47, line 7 through page 48, line 28) and antagonized by contacting the cell with an antibody against the cell fate control gene product or by expressing in the cell an antisense nucleic acid to the cell fate control gene transcript (as described in the specification at page 49, line 24 through page 50, line 2.

Additionally, the Examiner states that the present invention is not enabled for systems other than *Drosophila*, because the working examples in the specification are limited to *Drosophila* cell systems. However, "[t]here is no magical relation between the number of representative examples and the breadth of the claims" with respect to enablement. *In re Borkowski*, 164 U.S.P.Q. 642, 646 (C.C.P.A. 1970). The issue is not whether the specification discloses working examples of cell systems other than *Drosophila*, but whether teaches one of skill in the art how to practice the claimed invention in cell systems other than *Drosophila*. Applicants submit that the specification teaches one of skill in the art how to practice the claimed invention in such cell systems (*see*, *e.g.*, Section 5.9) and, given the conservation between the Notch pathway and cell fate control gene pathways among *Drosophila* systems and non-*Drosophila* systems, including mammalian systems (*see, e.g.*, Exhibits C and D attached hereto, which respectively demonstrate conservation in the Notch pathway among *Drosophila* and mammalian systems, as well as conservation in the activity of an exemplary cell fate control gene (*apterous*) among *Drosophila* and mammalian systems), one of skill in the art would conclude that the success of practicing the claimed

methods in the *Drosophila* working examples taught in the specification would be predictive of the ability to practice these methods in non-*Drosophila* systems.

Claims 1-5, 7-10, 13-15, 18-21, 24-34, 50-52, 63-66 and 69-83 are further rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement of *in vivo* methods. The Examiner states that the presently claimed invention is not enabled for *in vivo* methods because "fully successful" *in vivo* methods of transfection, particularly for long-term expression, are not available. In response, Applicants submit that a 100% percent success rate in gene delivery and perpetual expression of the Notch and cell fate control gene modulator is not required for practicing the claimed methods *in vivo*. Rather, all that is required is a rate delivery sufficient to achieve expression of the Notch and cell fate control gene modulators at a sufficient level and period of time to bring about a cell fate change, which can be achieved by gene delivery methods, for example as taught in the specification in Section 10 at pages 59-62. Further, the contrary to the Examiner's assertions, the specification does indeed recite a variety of applications for practicing the claimed methods *in vivo*, for example treatment of macular degeneration and cancer (*see* the specification at page 66, line 35 through page 67, line 1, and page Section 5.11.3 on page 63).

Applicants submit that because of (1) the teachings in the specification; (2) the high level of skill in the field of cellular and molecular biology; (3) the conservation between Notch and cell fate control gene activity among *Drosophila* and mammalian cells; and (4) the direction and guidance provided by the specification, one skilled in the art could routinely practice the claimed invention.

In view of the foregoing, Applicants submit that the rejections under 35 U.S.C. § 112, first paragraph, for lack of enablement have been obviated and should be withdrawn.

3. The Rejections Under 35 U.S.C. § 112, Second Paragraph, Should Be Withdrawn

Claims 1-5, 7-10, 13-15, 18-21, 24-34, 50-52, 63-66, and 69-83 are rejected under 35 U.S.C. § 112, second paragraph, allegedly as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner alleges that the claims are incomplete for omitting essential steps. In particular, the Examiner states that there is no step that creates a nexus

between step (c) and the preamble of claim 1, and that the claims are unclear because claim 1 "recited method steps within method steps."

In response, and without agreeing with the Examiner's rejection, Applicants have amended claim 1 to recite a conclusory phrase, thereby creating a nexus between step (c) and the preamble, and also to rephrase the language of steps (a) and (b) to avoid the recitation of a plurality of steps.

In view of the foregoing, Applicants submit that the rejections under 35 U.S.C. § 112, second paragraph, have been obviated and should be withdrawn.

CONCLUSION

Applicants respectfully request that the amendments and remarks of the present response be entered and made of record in the file of the above-captioned application. The pending claims fully meet all statutory requirements for patentability. Withdrawal of the Examiner's rejections, allowance and action for issuance are respectfully requested.

Applicants respectfully request that the Examiner call the undersigned at (212) 790-2247 if any questions or issues remain.

Respectfully submitted,

Date September 11, 2002

Adriane M. Antler 32,605
Adriane M. Antler (Reg. No.)

By:

Muna Abu-Shaar
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PENNIE & EDMONDS LLP
1155 Avenue of the Americas
New York, New York 10036-2711
(212) 790-9090

Enclosure

EXHIBIT A
MARKED UP COPY OF THE AMENDED CLAIMS
U.S. PATENT APPLICATION NO. 09/614,003
ATTORNEY DOCKET NO. 10910-092

1. (Twice amended) A method for altering the cell fate otherwise adopted by a cell comprising:

(a) [altering Notch pathway function in the cell by a method comprising] contacting the cell *in vitro* with, or administering to an organism comprising the cell, a modulator of Notch pathway function, thereby altering Notch pathway function in the cell;

(b) concurrently with step (a), [altering the function of a cell fate control gene pathway in the cell by a method comprising] contacting the cell *in vitro* with, or administering to an organism comprising the cell, a modulator of a cell fate control gene pathway function, wherein the cell fate control gene pathway is not the Notch pathway, thereby altering the function of a cell fate control gene pathway in the cell; and

(c) subjecting the cell to conditions that allow cell fate determination to occur,

thereby altering the cell fate otherwise adopted by the cell.

64. (Amended) The method according to claim 1 [or 53], wherein the cell fate produced by said method is apoptosis.

69. (Amended) The method according to claim 1 [or 53], wherein the cell fate that would have been otherwise adopted by said cell is apoptosis.

EXHIBIT B
CLEAN COPY OF CLAIMS AS PENDING FOLLOWING ENTRY OF AMENDMENTS MADE ON SEPTEMBER 11, 2002
U.S. PATENT APPLICATION NO. 09/614,003
ATTORNEY DOCKET NO. 10910-092

1. (Twice amended) A method for altering the cell fate otherwise adopted by a cell comprising:

(a) contacting the cell *in vitro* with, or administering to an organism comprising the cell, a modulator of Notch pathway function, thereby altering Notch pathway function in the cell;

(b) concurrently with step (a), contacting the cell *in vitro* with, or administering to an organism comprising the cell, a modulator of a cell fate control gene pathway function, wherein the cell fate control gene pathway is not the Notch pathway, thereby altering the function of a cell fate control gene pathway in the cell; and

(c) subjecting the cell to conditions that allow cell fate determination to occur,

thereby altering the cell fate otherwise adopted by the cell.

2. (Amended) The method according to claim 1 comprising contacting the cell *in vitro* with the modulator of Notch pathway function.

3. The method according to claim 2 which further comprises contacting the cell *in vitro* with an agonist of a cell fate control gene pathway function.

4. The method according to claim 2 which further comprises contacting the cell *in vitro* with an antagonist of a cell fate control gene pathway function.

5. (Amended) The method according to claim 1 comprising administering to an organism comprising the cell the modulator of Notch pathway function.

7. The method according to claim 1 comprising introducing into the cell one or more nucleic acids encoding an agonist of Notch pathway function and an agonist of a cell fate control gene pathway function such that the agonists are expressed by the cell.

8. The method according to claim 1 comprising introducing into the cell one or more nucleic acids encoding an agonist of Notch pathway function and an antagonist of a cell fate control gene pathway function such that the agonist and antagonist are expressed by the cell.

9. The method according to claim 1 wherein the agonist of Notch pathway function is a dominant-active Notch mutant.

10. The method according to claim 1 wherein the agonist is purified.

16. The method according to claim 1 comprising administering to an organism comprising the cell an antagonist of Notch pathway function and an agonist of a cell fate control gene pathway function.

17. The method according to claim 1 comprising administering to an organism comprising the cell an antagonist of Notch pathway function and an antagonist of a cell fate control gene pathway function.

18. The method according to claim 1 comprising introducing into the cell one or more nucleic acids encoding an antagonist of Notch pathway function and an agonist of a cell fate control gene pathway function such that the antagonist and agonist are expressed by the cell.

24. The method according to claim 1 in which the cell fate control gene encodes a transcription factor.

25. The method according to claim 24 in which the transcription factor is a homeodomain protein.

26. The method according to claim 25 in which the homeodomain protein is a Pax protein.

27. The method according to claim 26 in which the Pax protein is selected from the group consisting of human or mouse Pax-1, Pax-2, Pax-3, Pax-4, Pax-5, Pax-6, Pax-7, Pax-8 or Pax-9 and Drosophila Eyeless and Twin of Eyeless.

28. The method according to claim 25 in which the homeodomain protein is a Hox protein.

29. The method according to claim 28 in which the Hox protein is selected from the group consisting of Mammalian Hox A1-7, Hox A9-11 or HoxA13; Hox B1-9; Hox C4-6 or Hox C8-13; Hox D1, Hox D3-4 or Hox D8-13; and Drosophila Lab, Pb, Dfd, Scr, Antp, Ubx, Abd-A and Abd-B.

30. The method according to claim 25 in which the homeodomain protein is selected from the group consisting of a DLX protein, LIM homeodomain protein, PBC protein, MEINOX protein, POU protein, PTX protein and NKX protein.

31. The method according to claim 24 in which the transcription factor is selected from the group consisting of a Vestigial protein, MADS domain protein, bHLH protein, SOX protein and T-box protein.

32. The method according to claim 1 in which the cell fate control gene encodes a signaling molecule.

33. The method according to claim 32 wherein the signaling molecule is selected from the group consisting of a Hedgehog protein, a WNT protein, and a TGF /BMP protein.

34. The method according to claim 1 which further comprises expanding the cell by subjecting the cell to cell growth conditions to produce a population of cells.

50. (Amended) The method according to claim 1 or 5 wherein the altering of cell fate is a change in tissue or organ type.

51. The method according to claim 1 wherein the cell is a mammalian cell.

52. The method according to claim 51 wherein the cell is a human cell.

63. (Amended) The method according to claim 1 wherein the modulator of Notch pathway function and the modulator of the cell fate control gene pathway function are purified.

64. (Amended) The method according to claim 1, wherein the cell fate produced by said method is apoptosis.

65. The method according to claim 63 wherein the cell is a human cell.

66. The method according to claim 64 wherein the cell is a cancer cell.

69. (Amended) The method according to claim 1, wherein the cell fate that would have been otherwise adopted by said cell is apoptosis.

70. The method of claim 2, wherein the modulator of Notch pathway function is an antagonist of Notch pathway function.

71. The method of claim 70, which comprises contacting the cell *in vitro* with the modulator of a cell fate control gene pathway function.

72. The method of claim 71, wherein the modulator of the cell fate control gene pathway function is an antagonist of the cell fate control fate control gene pathway function.

73. The method of claim 70, which comprises administering to an organism comprising the cell the modulator of a cell fate control gene pathway function.

74. The method of claim 73, wherein the modulator of the cell fate control gene pathway function is an antagonist of the cell fate control fate control gene pathway function.

75. The method of claim 5, wherein the modulator of Notch pathway function is an antagonist of Notch pathway function.

76. The method of claim 75, which comprises contacting the cell *in vitro* with the modulator of a cell fate control gene pathway function.

77. The method of claim 76, wherein the modulator of the cell fate control gene pathway function is an antagonist of the cell fate control fate control gene pathway function.

78. The method of claim 75, which comprises administering to an organism comprising the cell the modulator of a cell fate control gene pathway function.

79. The method according to claim 70 comprising contacting the cell *in vitro* with an antagonist of Notch pathway function.

80. The method according to claim 72 comprising introducing into the cell one or more nucleic acids encoding an antagonist of Notch pathway function and an antagonist of a cell fate control gene pathway function such that the antagonists are expressed by the cell.

81. The method according to claim 70 wherein the antagonist of Notch pathway function is a dominant-negative Notch mutant.

82. The method according to claim 70 wherein the antagonist is purified.

83. The method according to claim 70 or 75 wherein the altering of cell fate is a change in tissue or organ type.

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Conservation of the Notch signalling pathway in mammalian neurogenesis

José Luis de la Pompa[1,2,*], Andrew Wakeham[1,2], Kristen M. Correia[3], Enrique Samper[1,2], Stephen Brown[4], Renato J. Aguilera[4], Toru Nakano[5], Tasuku Honjo[5], Tak W. Mak[1,2], Janet Rossant[6,7] and Ronald A. Conlon[3]

[1]Amgen Institute, 620 University Avenue, Toronto, Ontario, Canada M5G 2C1
[2]Ontario Cancer Institute, and Departments of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario, Canada M5G 2C1
[3]Department of Genetics, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, Ohio 44106-4955, USA
[4]Department of Molecular, Cell, and Developmental Biology, University of California at Los Angeles, Los Angeles, California 40095-1606, USA
[5]Department of Medical Chemistry, Faculty of Medicine, Kyoto University, Kyoto 606, Japan
[6]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario, Canada M5G 1X5
[7]Departments of Molecular and Medical Genetics, and Obstetrics and Gynecology, University of Toronto

*Author for correspondence (e-mail: Jose.delaPompa@amgen.com)

SUMMARY

The *Notch* pathway functions in multiple cell fate determination processes in invertebrate embryos, including the decision between the neuroblast and epidermoblast lineages in *Drosophila*. In the mouse, targeted mutation of the Notch pathway genes *Notch1* and *RBP-Jk* has demonstrated a role for these genes in somite segmentation, but a function in neurogenesis and in cell fate decisions has not been shown. Here we show that these mutations lead to altered expression of the Notch signalling pathway homologues *Hes-5*, *Mash-1* and *Dll1*, resulting in enhanced neurogenesis. Precocious neuronal differentiation is indicated by the expanded expression domains of *Math4A*, *neuroD* and *NSCL-1*. The *RBP-Jk* mutation has stronger effects on expression of these genes than does the *Notch1* mutation, consistent with functional redundancy of *Notch* genes in neurogenesis. Our results demonstrate conservation of the Notch pathway and its regulatory mechanisms from fly to mouse, and support a role for the murine Notch signalling pathway in the regulation of neural stem cell differentiation.

Key words: mouse, neurogenesis, Notch pathway

INTRODUCTION

Neurogenesis in vertebrates occurs by the regulated withdrawal from the cell cycle of a homogeneous population of progenitor cells in the neural tube (McConnell, 1981). For example, in the mammalian cerebral cortex, prospective neurons individually cease division, migrate centrifugally and differentiate. This process is reiterated throughout development, generating radially arranged layers of neurons, with the last-born neurons in the outermost layer (McConnell, 1995). Clearly, this process has to be controlled both spatially and temporally, in order to generate the correct number of neurons in different regions of the developing central nervous system (CNS). It is thus essential to understand the mechanisms that regulate the birth of neurons in the mammalian CNS.

In *Drosophila*, neurogenesis is initiated by the separation of neural progenitors (neuroblasts) from progenitors of the epidermis (epidermoblasts). Prior to this separation, neuroblasts and epidermoblasts are intermingled in the neurogenic ectoderm (Campos-Ortega, 1993). Each cell in this region has the potential to become either a neuroblast or an epidermoblast and has to choose between these developmental fates (Technau and Campos-Ortega, 1986). Cell-to-cell interactions involving direct contacts between neighboring cells are essential for the proper separation of these two lineages. Thus, a prospective neuron inhibits its neighbors from also developing into neurons, a mechanism termed lateral inhibition (Heitzler and Simpson, 1991; Greenwald and Rubin, 1992). Lateral inhibition also regulates the number of cells that become neurons, as well as their spatial arrangement. The products of the so-called neurogenic genes participate in this cell communication process. A typical neurogenic phenotype is defined by lack-of-function mutations that cause the expansion of the nervous system at the expense of the epidermis (Lehmann et al., 1981). The genes *Delta* (*Dl*), *Notch* (*N*), *Suppressor of Hairless* (*Su(H)*) and the *Enhancer of split* (*E(spl)*) complex, belong to the neurogenic group.

Notch encodes for a large membrane-spanning protein (Wharton et al., 1985) that acts as receptor for the membrane-bound ligands Delta (Vässin et al., 1987) and Serrate (Fleming et al., 1990). Genes related to *Notch* and *Delta* have been identified in several different species. All known ligands for Notch-related receptors are membrane-bound (Artavanis-Tsakonas et al., 1995; Greenwald, 1994), and biological assays indicate that Notch signalling occurs only between cells that are in direct contact with each other (Heitzler and Simpson, 1991). At present, there are two models of Notch signal transduction to the nucleus. In the first model, ligand binding is thought to cause the translocation of the transcription factor Su(H) from the cytoplasm to the nucleus (Fortini and Artavanis-Tsakonas,

1994). In the second model, ligand binding is thought to induce proteolytic processing of Notch and translocation of a fragment of Notch to the nucleus, where it binds to and activates Su(H) (Jarriault et al.,1995). A recent study in *Drosophila* indicates that differential subcellular localization of Su(H) is not essential for its function (Gho et al., 1996). Which model is correct remains unresolved.

Genetic and molecular data have shown that the genes of the *E(spl)* complex are the first target for Su(H) after Notch signalling (Lecourtois and Schweisguth, 1995; Bailey and Posakony, 1995). In vertebrates, homologues to all these Notch pathway genes exist, including *Delta* (Chitnis et al., 1995; Henrique et al., 1995; Bettenhausen et al., 1995), *Serrate* (Lindsell et al., 1995; Myat et al., 1996), *Notch1-4* (Weinmaster et al., 1991, 1992; Lardelli et al., 1994; Uyttendaele et al., 1996), *RBP-Jk* (Recombination signal sequence Binding Protein for *Jk* genes) homologue of *Su(H)* (Furukawa et al., 1992; Schweisguth and Posakony, 1992) and *Hes-1-5* (*Hairy* and *Enhancer of split* homologues; Sasai et al., 1992; Takebayashi et al., 1995). Moreover, these genes are expressed in the CNS as well as other regions of the body (Chitnis et al., 1995; Henrique et al., 1995; Bettenhausen et al., 1995; Franco del Amo, 1992; Reaume et al., 1992; Sasai et al., 1992; Akazawa et al., 1992). Experimental studies in the embryonic chick retina (Austin et al., 1995), the *Xenopus* embryonic CNS (Chitnis et al., 1995) and mammalian cells in culture (Nye et al., 1994) have suggested that the Notch signalling pathway functions in vertebrate neurogenesis. However, the phenotypic analysis of mouse mutations in either *Notch1* (Swiatek et al., 1994; Conlon et al., 1995) or its putative downstream effector *RBP-Jk* (Oka et al., 1995), did not reveal a role for the Notch pathway in neurogenesis, largely because both mutations cause embryonic death around day 9 of development, just as neuronal differentiation is beginning.

In this paper, we present the results of an investigation of the role of Notch signalling in mouse neurogenesis. First, we show that the RBPJK protein of the mouse embryo is predominantly localized to the nucleus and shows no obvious variations in cellular localization in *Notch1* mutants. Secondly, we show that *Hes-5*, *Mash-1* and *Dll1* are targets of the Notch signalling pathway. Thirdly, we show that more cells express early neuronal differentiation markers in *RBP-Jk* and *Notch1* mutants, suggesting that activation of Notch signalling negatively regulates the formation of neurons in the neural tube of the mouse. Lastly, we demonstrate that the *RBP-Jk* mutation causes a more severe neurogenic phenotype than the *Notch1* mutation, indicating that there may be functional redundancy of the different Notch proteins of the mouse. This provides further evidence that the Notch signalling pathway, its regulatory mechanisms and its role in neurogenesis are conserved from fly to vertebrates.

MATERIALS AND METHODS

Genotyping

RBP-Jk and *Notch1* mutant embryos were obtained by mating females and males heterozygous for *RBP-Jk* (Oka et al., 1995) or *Notch1* (Conlon et al., 1995), targeted mutations, respectively. Embryos were genotyped by PCR analysis of the yolk sacs. Primers and conditions were as described previously.

Gen ration of anti-RBPJK-specific antis rum

Polyclonal antibodies were raised against a RBPJK polypeptide containing the first 276 aa of the protein. The RBP (1-276) polypeptide was produced in BL-21 (protease-deficient) bacteria using the pET-15b expression vector (Novagen). Clones containing the RBP expression construct were induced with IPTG to express the recombinant protein at high levels. Recombinant protein was purified to homogeneity by passage over nickel-coated beads (Novagen). Purified protein (1-2 μg/boost) was used to inject a New Zealand white rabbit. The antiserum obtained was evaluated by western blot analysis of crude lymphocyte nuclear extracts (data not shown). For the purification of the antiserum, the technique described by Hall et al. (1984) was used: recombinant RBP protein (500 μg) was separated on a 15% SDS-PAGE gel, transferred to a PVDF membrane and detected by Ponceau Red staining. The RBP strip was excised from the membrane and incubated with 2 ml of antiserum. Anti-RBP-specific antibodies were eluted at pH 2.6 in a glycine buffer and subsequently neutralized with Tris pH 7.5.

Embryo extracts and western blot analysis

Embryos were dissected in ice-cold PBS and frozen immediately. Extracts were prepared using the method described by Lee et al (1988). 25 μg of extract was loaded per lane. Affinity-purified polyclonal anti-RBPJK antibodies were used at a 1/500 dilution. Staphylococcus protein A coupled to horseradish peroxidase (Sigma) was used at a dilution of 1/10,000. Antibody bound to proteins were visualized using the LumiGLO chemiluminescence substrate kit (Kirkegaard and Perry Laboratories) as described by the manufacturer. To verify that equivalent amounts of extracts were loaded in each lane, blots were stripped and reprobed with antiserum (1/2,000) against the ubiquitous protein nucleolin (Miranda et al., 1995).

Immunohistochemistry

Embryos were isolated in ice-cold PBS, fixed in 4% paraformaldehyde for 3 hours, dehydrated, embedded in wax and sectioned at 5 μm. Affinity-purified anti-RBPJK antiserum was used at a 1/50 dilution. A biotinylated secondary antibody against rabbit IgG and avidin-conjugated peroxidase (Vector Laboratories) were used for immunostainings. A protocol described by Trumpp et al. (1992) was used. Briefly, rehydrated sections were incubated for 2 hours at room temperature with the primary antibody, 1 hour with goat anti-rabbit IgG and 1 hour with avidin-peroxidase complex at room temperature. The signal was visualized in 30-60 minutes by an HRP reaction (Vector Laboratories) using diaminobenzidine (DAB, 1 mg/ml in 0.1 M TrisHCl, pH 7.5) and hydrogen peroxide (0.03% final) as substrates. To enhance the signal, $NiCl_2$ (0.04% final) was used in the developer cocktail.

Northern blot analysis

Total RNA was extracted from embryonic day 8.5-9.0 (E8.5-E9.0) whole embryos using Trizol (Life Technologies). 20 μg of total RNA was electrophoresed on a formaldehyde/1% agarose gel and transferred to a nylon membrane (Hybond N^+, Amersham). Full-length *Hes-1*, *Hes-3* and *Hes-5* cDNAs were ^{32}P-labeled and used as probes for hybridization at 65°C in Church and Gilbert buffer. Filters were subsequently stripped and rehybridized with a mouse *β-actin* probe.

Whole-mount in situ hybridization

Embryos were isolated in ice-cold PBS, fixed overnight in 4% paraformaldehyde and processed for whole-mount in situ hybridization following described procedures (Conlon and Hermann, 1993; modified following Koop et al., 1996).

Hist l gy

After whole-mount in situ hybridization embryos were postfixed overnight in 4% paraformaldehyde, dehydrated, cleared in xylene for 15 minutes, embedded in wax and sectioned at 10 μm.

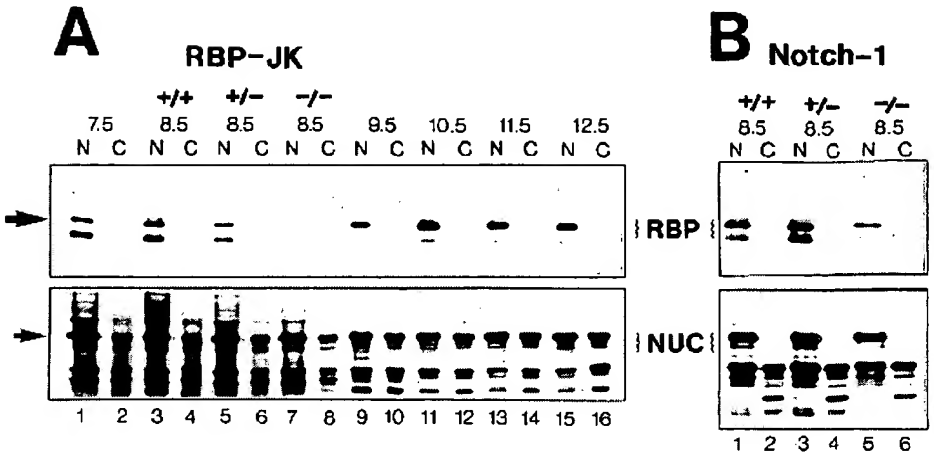

Fig. 1. Nuclear localization of RBPJK throughout embryogenesis. (A) Western blot showing RBPJK expression during embryogenesis (Lanes 1-16). RBPJK is always present in the nuclear fraction (N; lanes 1, 3, 5, 9, 11, 13, 15) and never in the cytoplasmic one (C; lanes 2, 4, 6, 10, 12, 14, 16). Decreased RBPJK expression is observed in heterozygous (+/–) E8.5 *RBP-Jk* embryos (lane 5). No signal is observed in E8.5*RBP-Jk* homozygous (–/–) mutant embryos (Lane 7). (B) Western blot showing nuclear localization of RBPJK in E8.5 wild-type (+/+; lane 1), *Notch1* heterozygous (+/–; lane 3) and *Notch1* homozygous (–/–) mutant embryos (lane 5). The big arrow in the upper panels in A,B indicates full-length RBPJK protein (~60×10^3 M_r; RBP) and the small arrow in the lower panels points to the nucleolin protein (~105×10^3 M_r; NUC). The lower band in the RBPJK blot is most likely the result of alternative splicing of the *RBP-Jk* primary transcript (Kawaichi et al., 1992) or a proteolytic fragment of RBPJK. Nucleolin is known to undergo autoproteolysis and, in addition to being present in the nucleus, is also found in the cytoplasm, mostly in degraded form (as in B; Miranda et al., 1995).

RESULTS

RBPJK is nuclear in wild-type and *Notch1* mutant embryos

To attempt to determine whether nuclear localization of RBPJK varies according to activity of the Notch pathway, polyclonal antibodies raised against RBPJK were used to study the expression and subcellular localization of this protein in the mouse embryo. Western blot analysis reveals that a 60×10^3 M_r RBPJK protein is present in the nucleus throughout embryogenesis and not readily detectable in the cytoplasm component (Fig. 1A; lanes 1, 3, 5, 9, 11, 13 and 15). The specificity of the antiserum is demonstrated by the absence of signal in extracts from *RBP-Jk* mutant embryos (Fig. 1A; lane 7). To determine if the absence of the Notch1 receptor would affect the subcellular localization of RBPJK, we performed western blot analysis of protein extracts from *Notch1* mutant embryos and wild-type littermates. In both wild-type (Fig. 1B; lanes 1, 3) and *Notch1* mutant embryos (Fig. 1B; lane 5), RBPJK is always present in the nucleus.

Immunohistochemical analysis of sections of E8.5 wild-type embryos also revealed widespread RBPJK nuclear staining in the neural tube and paraxial mesoderm (Fig. 2A-C). This staining was absent in *RBP-Jk* mutant embryos (Fig. 2F). Staining of E8.5 *Notch1* embryos also revealed a nuclear localization of RBPJK (Fig. 2D,E). There was no clear change in the nuclear localization of RBPJK in wild-type or *Notch1* mutant embryos that could be related to areas of activity of the Notch pathway. Staining of embryos with the anti-RBPJK monoclonal antibody T6719 (Hamaguchi et al., 1992) supported these results (data not shown).

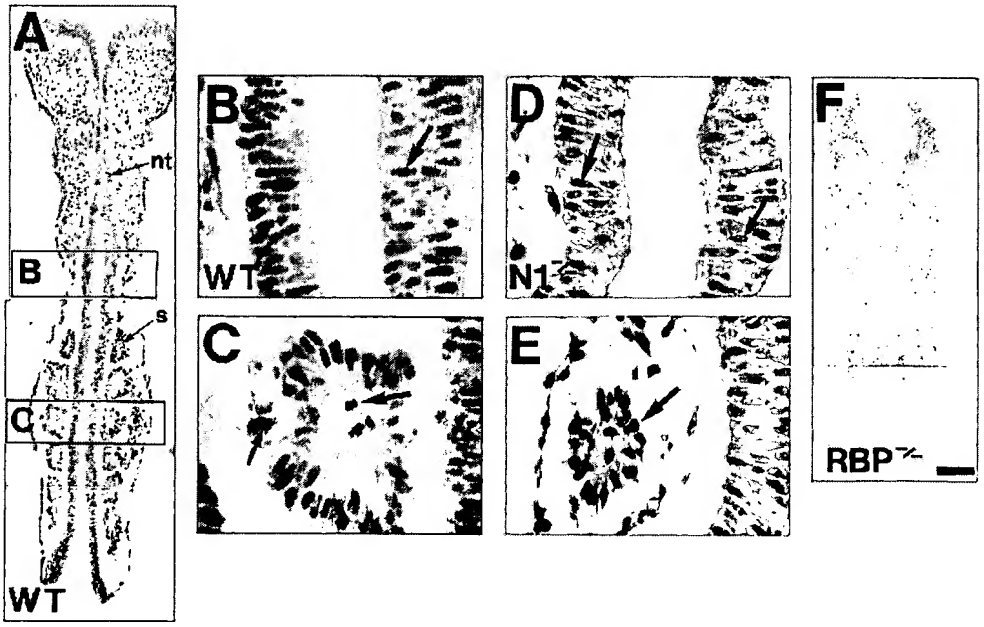

Fig. 2. RBPJK expression in E8.5 embryos. (A) Wild-type embryo, section through the brain, neural tube and somitic region. Nuclei expressing RBPJK appear dark. (B,C) Details showing RBPJK nuclear staining in neural tube (arrow in B) and somite cells (larger arrow in C). (D,E) *Notch1* homozygous mutant embryo. Details showing RBPJK nuclear staining in the neural tube (larger arrow in D) and somite cells (arrow in E). The small arrows in C,D point to the nucleolus that does not express RBPJK. (F) *RBP-Jk* homozygous mutant embryo. No signal is detected. All sections are horizontal. nt, neural tube; s, somites. Bar, 80 μm in A,F; 30 μm in B-E.

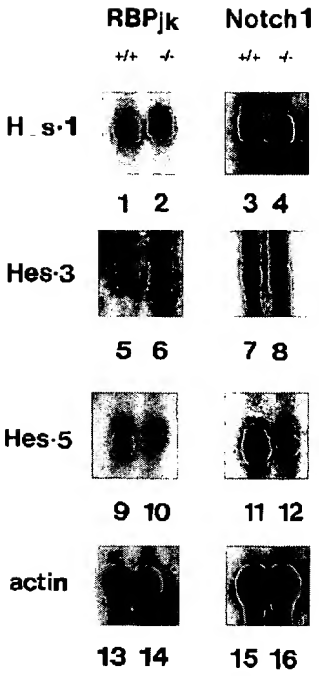

Fig. 3. *Hes-5* expression in *RBPJk* and *Notch1* mutant embryos: northern blot analysis of E9.0 embryos. Total RNA from wild-type (lanes 1, 3, 5, 7, 9, 11, 13, 15), homozygous mutant *RBP-Jk* (lanes 2, 6, 10, 14) and *Notch1* (lanes 4, 8, 12, 16) embryos was hybridized with *Hes-1* (lanes 1-4), *Hes-3* (lanes 5-8) and *Hes-5* (lanes 9-12) probes. Expression of *Hes-5* is strongly down-regulated in *RBP-Jk* mutant embryos (lane 10) and to a lesser extent in *Notch1* mutants (lane 12). A *β-actin* probe (lanes 13-16) was used as a control for sample loading.

Down-regulation of *Hes-5* and up-regulation of *Mash-1* expression in *RBP-Jk* and *Notch1* mutant embryos

In *Drosophila*, the spatiotemporal expression pattern of the basic Helix-Loop-Helix (bHLH) transcription factors encoded by the *E(spl)* complex, suggests that they accumulate in response to Notch signalling activity (Jennings et al., 1995). Studies in vertebrates indicate that the related *Hes* genes are candidates to be positively regulated by RBPJK (Jarriault et al., 1995). We examined the expression of *Hes* genes by northern blot and whole-mount in situ hybridization (Figs 3, 4 and data not shown). Only *Hes-1*, *Hes-3* and *Hes-5* are detectably expressed at E8.5-9.0. Levels of *Hes-1* and *Hes-3* expression were not changed in *RBP-Jk* and *Notch1* mutant embryos and the spatial distribution of transcripts was not affected (Fig. 3; lanes 2, 4, 6, 8; and data not shown). In contrast, *Hes-5* expression was almost undetectable in northern blots from *RBP-Jk* mutants (lane 10) and reduced by half in *Notch1* mutants (lane 12). *Hes-5* is normally expressed in a stripe in the midbrain region, two stripes in the hindbrain region and along the neural tube, as well as in the primitive streak, and in two pairs of stripes in the presomitic mesoderm (Fig. 4A). Expression of *Hes-5* was severely reduced in all its expression domains in *RBP-Jk* mutant embryos (Fig. 4B). *Hes-5* expression was also down-regulated in *Notch1* mutants (Fig. 4C), although not so dramatically, in agreement with the

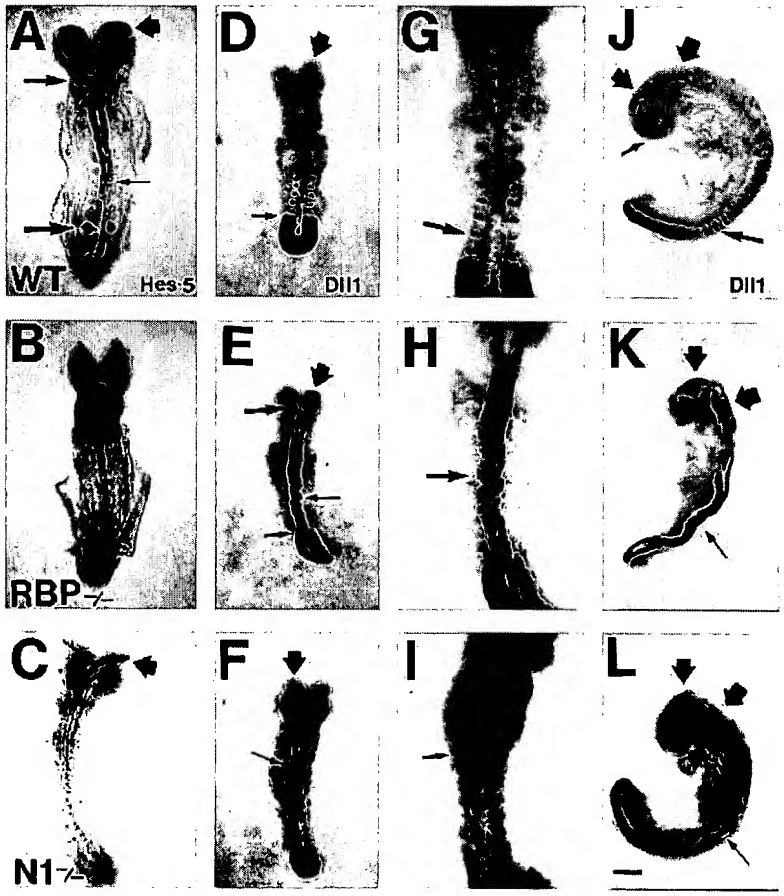

Fig. 4. Down-regulation of *Hes-5* and up-regulation of *Dll1* expression in *RBP-Jk* and *Notch1* mutant embryos. Whole-mount in situ hybridization of E8.5 wild-type (A,D,G); E9.0 wild-type (J); E8.5 *RBP-Jk* (B,E,H); E9.0 *RBP-Jk* (K); E8.5 *Notch1* (C,F,I) and E9.0 *Notch1* (L) embryos. (A-I) Embryos are viewed dorsally, anterior is at the top; (J-L) embryos are oriented laterally. (A) *Hes-5* expression in a wild-type embryo. Signal is detected in midbrain (thick arrow), hindbrain (arrow), neural tube (small arrow) and presomitic mesoderm (big arrow). (B) *Hes-5* expression in a *RBP-Jk* mutant embryo. Signal is strongly down-regulated. (C) *Hes-5* expression in a *Notch1* mutant embryo. Signal is downregulated, although some expression is detected in the midbrain (arrow). (D,G,J) *Dll1* expression in a wild-type embryo. (D) Expression is detected in the midbrain (thick arrow) and presomitic mesoderm (small arrow). (G) The arrow points to expression in the posterior region of a somite. (J) Signal is detected in the forebrain region (small arrow), midbrain (thick arrow), hindbrain (big arrow) and posterior region of the somites (arrow). (E,H,K) *Dll1* expression in a *RBP-Jk* mutant embryo. (E) Expression is detected in the midbrain (thick arrow); one stripe in the hindbrain (arrow), neural tube (thin arrow) and the presomitic mesoderm (small arrow). Signal is strongly up-regulated in the neural tube and brain. Note the striking similarity with the wild-type expression of *Hes-5*, shown in A. (H) The arrow points to up-regulated *Dll1* expression in the neural tube. (K) Up-regulated *Dll1* expression is detected in the midbrain (thick arrow), hindbrain (big arrow) and neural tube (thin arrow). (F,I,L) *Dll1* expression in a *Notch1* mutant embryo. (F) Up-regulated expression is observed in the midbrain (thick arrow) and neural tube (thin arrow). Expression is also observed in the presomitic mesoderm and in the somites (arrow in I). (L) Up-regulated expression is detected in the midbrain (thick arrow), hindbrain (big arrow) and along the neural tube (thin arrow). Bar, 40 μm in G-I; 80 μm in the rest.

northern blot analysis. These results are consistent with *RBP-Jk* and *Notch1* acting in a common pathway to activate *Hes-5* expression in the embryo.

The results obtained above are analogous to what is known of the *Drosophila* Notch pathway. In *Drosophila*, loss of *E(spl)* activity leads to an upregulation of genes of the *achaete-scute* (*ac-sc*) proneural class and, consequently, to an excess of neuroblasts (Skeath and Carroll, 1992). One might predict that loss of *Hes-5* signalling in *RBP-Jk* and *Notch1* mutants would lead to deregulated expression of *Mash-1*, a mouse homologue of *ac-sc* (Guillemot and Joyner, 1993). *Mash-1* is expressed at E8.5 in the anterior region of the neural tube (Fig. 5A), and in scattered cells of the midbrain region from which the first neurons of the CNS emerge (Fig. 5C). In *RBP-Jk* mutant embryos at E8.5, *Mash-1* expression was increased in intensity and extent in the forebrain, midbrain (Fig. 5B) and hindbrain regions (Fig. 5D), and in the anterior part of the neural tube (Fig. 5B,D). At E9.0, wild-type *Mash-1* expression is restricted to the dorsal midbrain and to two patches at either side of the otic vesicle (Fig. 5E,G,I). In the *RBP-Jk* and *Notch1* mutants at E9.0, *Mash-1* midbrain and hindbrain expression was found to be more intense (Fig. 5F,H,J,K), and extended over a larger area of the dorsal and ventral regions of the neural tube (Fig. 5F,H). The latter may correspond to neural-crest-derived precursors of sympathetic ganglia. Taken together, these observations are consistent with *Hes-5* down-regulation of *Mash-1* expression in the midbrain, hindbrain and neural tube.

Histological analysis of E9.0 wild-type and mutant embryos revealed that *Mash-1* expression was restricted to the subventricular zone of the dorsal midbrain in wild-type embryos (Fig. 5L, see also Guillemot and Joyner, 1993). In contrast, *Mash-1* expression domain was expanded to the ventricular zone in the midbrain of *RBP-Jk* and *Notch1* mutant embryos (Fig. 5M,N), suggesting that an excess of committed neuronal precursors were generated in mutant embryos.

Neural tube expression of *Dll1* is increased in *RBP-Jk* and *Notch1* mutants

In lateral inhibition models, Notch ligand expression is responsive to the state of Notch activation (Heitzler and Simpson, 1991; Wilkinson et al., 1994; Heitzler et al., 1996; see Fig. 7A and Discussion). Thus, cells that are stimulated by the ligand down-regulate expression of the ligand itself. Inactivation of the Notch receptor pathway should lead to increased expression of the ligand. To determine if ligand expression is responsive to the Notch pathway in the mouse, *Dll1* expression was examined in *RBP-Jk* and *Notch1* mutant embryos. *Dll1* is normally expressed in the primitive streak and presomitic mesoderm throughout embryogenesis (Bettenhausen et al., 1995). In E8.5 and E9.0 embryos, it is also expressed in the posterior of each somite (Fig. 4D,G,J). In the neural tube, *Dll1* is expressed in individual, isolated cells in a basal position in the neural epithelium, in cells that are thought to be committed neuronal precursors (Bettenhausen et al., 1995; Henrique et al., 1995). In *RBP-Jk* and *Notch1* mutant embryos, the abundance of *Dll1* RNA was not altered as determined by northern blot analysis (not shown). However, there were dramatic changes in the spatial distribution of *Dll1* mRNA as determined by in situ hybridization. In contrast to wild-type embryos, where *Dll1* was expressed in scattered cells in the neural tube, in *RBP-Jk* mutant embryos, *Dll1* was expressed in all cells of the presumptive spinal cord (Fig. 4E,H,K). *Dll1* expression in the neural tube was upregulated in *Notch1* embryos as well, although to not as great an extent (Fig. 4F,I,L). The expression of *Dll1* in *RBP-Jk* mutants was strikingly similar to normal *Hes-5* expression in wild-type embryos (cf Fig. 4A with Fig. 4E), suggesting that *Hes-5* expression represses *Dll1* transcription in these regions. In contrast, *Dll1* expression in the primitive streak and presomitic mesoderm was not changed in the mutant embryos (Fig. 4E,F) and *Dll1* expression in the somites was lost in *RBP-Jk* mutants (Fig. 4H).

Enhanced neuronal differentiation in *RBP-Jk* and *Notch1* mutant embryos

The up-regulated expression of *Mash-1* and *Dll1* in the CNS of *RBP-Jk* and *Notch1* mutants is very reminiscent of events in *Drosophila* neurogenesis where deregulated expression of *Dl* and *E(spl)* in *Notch* mutants leads to an excess of neuroblast differentiation. To determine whether these events led to an excess of neuronal differentiation in the mouse, the expression of three bHLH transcription factors that are expressed in early differentiating neurons, *Math4A*, *neuroD* and *NSCL-1*, was studied. *Math4A* is related to the *Drosophila* proneural gene *atonal* (Gradwohl et al., 1996). In E9.0 wild-type embryos, *Math4A* is expressed in neuronal precursors in the midbrain and ventral spinal cord (Gradwohl et al., 1996; Fig. 6A). In *RBP-Jk* (Fig. 6B), and to a lesser extent in *Notch1* mutant embryos (Fig. 6C), *Math4A* expression was increased in these regions. *neuroD* expression is specifically restricted to the developing trigeminal ganglia at E9.0 (Lee et al., 1995 ; Fig. 6D). In *RBP-Jk* and *Notch1* mutant embryos, *neuroD* was expressed at high levels in the trigeminal ganglion, and was ectopically expressed in the midbrain and the anterior spinal cord (Fig. 6E,F). Interestingly, *neuroD* expression in the midbrain and spinal cord overlapped with that of up-regulated *Mash-1* (compare Figs 5F versus 6E or 5H versus 6F), suggesting that *Mash-1* may positively regulate *neuroD* transcription in these regions. *NSCL-1* also shows a restricted expression and, at E9.0, is transcribed in scattered cells in the anterior midbrain (Begley et al., 1992; Fig. 6G). In *RBP-Jk* mutant embryos, *NSCL-1* expression was increased in the midbrain region, and was ectopically expressed in the trigeminal ganglion and in the caudal neural tube (Fig. 6H). *Notch1* mutant embryos also showed increased and ectopic expression of *NSCL-1* (Fig. 6I), although the increase in expression was less dramatic. Increased expression of these three neuronal differentiation markers in *RBP-Jk* and *Notch1* mutant embryos, confirmed that an excess of committed neuronal precursors were generated at E9.0 in the mutants. This is the first description of a neurogenic phenotype resulting from the disruption of the Notch signalling pathway in the mouse.

DISCUSSION

Target gen s of Notch signalling and neurogenesis

In this report, we have provided evidence showing that many changes in gene expression occur in *RBP-Jk* and *Notch1* mutants that reveal striking similarities with the Notch sig-

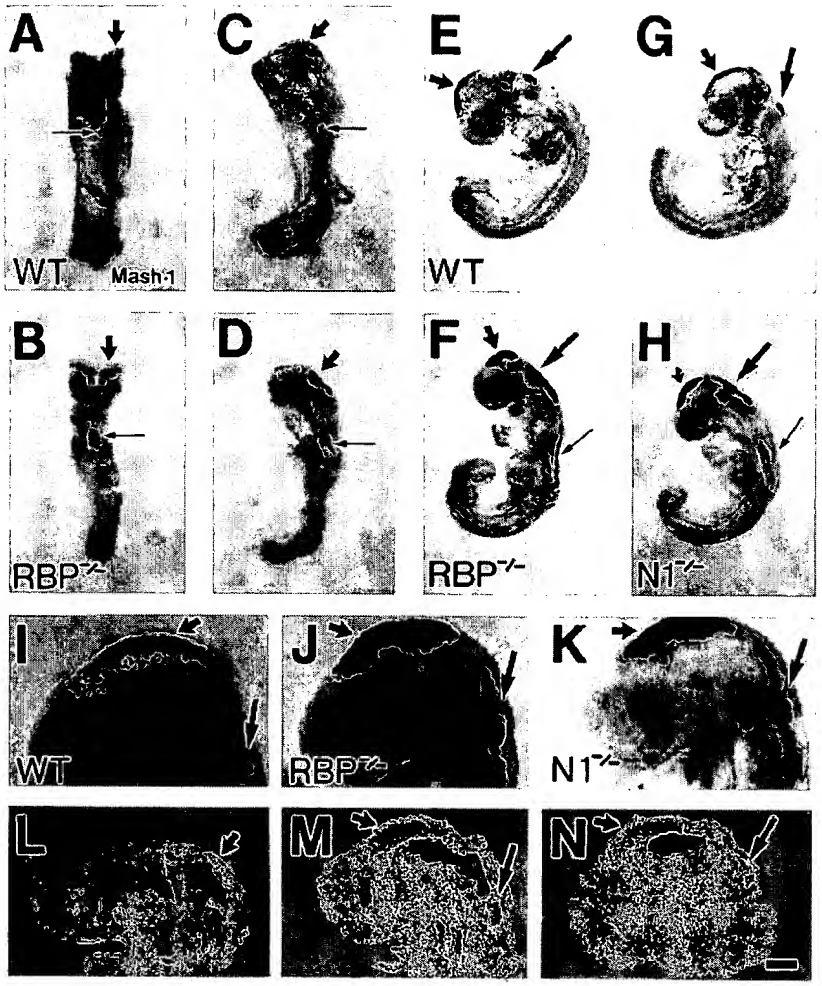

Fig. 5. *Mash-1* expression is up-regulated in *RBP-Jk* and *Notch1* mutant embryos. Whole-mount in situ hybridization of E8.5 wild-type (A,C); E9.0 wild-type (E,G,I,L); E8.5 *RBP-Jk* (B,D); E9.0 *RBP-Jk* (F,J,M) and E9.0 *Notch1* (H,K,N) embryos. (A,B) Embryos are viewed dorsally. (C-N) Embryos are oriented laterally. (A,C) *Mash-1* expression in a E8.5 wild-type embryo. Signal is detected in the anterior neural tube region (thin arrow) and in the midbrain (thick arrow). (B,D) *Mash-1* expression in a E8.5 *RBP-Jk* embryo. Up-regulated expression is observed in the midbrain/hindbrain region and in the anterior neural tube (thick and thin arrows, respectively). (E,G) *Mash-1* expression in an early (E) and more advanced (G) E9.0 wild-type embryo. (E,G) Signal is observed in the midbrain (thick arrow) and in two patches flanking the otic vesicle (arrow points to the posterior patch). (F) *Mash-1* expression in an early E9.0 *RBP-Jk* mutant embryo. Signal is up-regulated in the midbrain (thick arrow), hindbrain (big arrow) and both dorsal and ventral neural tube (thin arrow). (H) *Mash-1* expression in a E9.0 *Notch1* embryo. Stage is comparable to the wild-type embryo shown in G. Up-regulation of signal is noted in the midbrain (bent arrow), hindbrain (big arrow) and neural tube (thin arrow). (I) Detail of *Mash1* expression in the midbrain region (thick arrow) and in a patch posterior to the otic vesicle (arrow) in a E9.0 wild-type embryo. (J,K) Detail of up-regulated *Mash1* expression in the midbrain and hindbrain regions (thick and big arrow, respectively) of E9.0 *RBP-Jk* (J) and *Notch1* (K) mutant embryos. (L-N) Histological sections of the embryos shown in (I-K). (L) *Mash1* expression is restricted to the dorsal most region of the midbrain in a E9.0 wild-type embryo (arrow). (M,N) *Mash1* expression is enhanced and its expression domain expanded dorsoventrally to the ventricular zone in the midbrain (thick arrow) and hindbrain region (big arrow) of *RBP-Jk* (M) and *Notch 1* (N) mutant embryos. Bar, 80 μm in A-H; 8 μm in I-N.

nalling pathway deduced in *Drosophila*. Thus, studies in *Drosophila* have suggested that the genes of the *E(spl)* complex are directly regulated by the Notch pathway (Bailey and Posakony, 1995; Jennings et al., 1995; Lecourtois and Schweisguth, 1995; Furukawa et al., 1995). In mammalian systems, RBPJK has been shown to bind to the regulatory sequences and activate transcription of *Hes-1* in vitro, in combination with the intracellular domain of Notch (Jarriault et al., 1995). However, we could not detect any change in *Hes-1* expression in *RBP-Jk* and *Notch1* mutants, suggesting that the contribution of these genes to the regulation of *Hes-1* in the early embryo must be minimal. In contrast, the expression of another *Hes* family member, *Hes-5*, was reduced in *Notch1* mutants and almost completely eliminated in *RBP-Jk* mutants, indicating that *Hes-5* is highly responsive to Notch signalling. Potential RBPJK-binding sites exist in the promoter of *Hes-5* (data not shown), suggesting that RBPJK may directly regulate the transcription of *Hes-5*. A putative *Hes-5* target is *Mash-1*, whose expression domain is expanded in *RBP-Jk* and *Notch1* mutants. Thus, in *Drosophila*, low levels of *E(spl)* expression imply high *ac-sc* expression (Skeath and Carroll, 1992) and, in the mouse, low *Hes-5* expression correlates with enhanced *Mash-1* expression expanding over a larger CNS region. Interestingly, mutation of *Hes-1* leads also to up-regulation of *Mash-1* (Ishibashi et al., 1995), indicating that alternate pathways for activating *Mash-1* must exist.

Dll1 expression is also highly responsive to mutations in genes of the Notch pathway. In this case, however, the expression of *Dll1* in the neural tube is increased in both *RBP-Jk* and *Notch1* mutants. This result implies that *Dll1* expression is normally repressed by signalling through the Notch pathway.

We have no data that bear on whether this regulation is direct or indirect, but the strength of the response implies that this is an important regulatory interaction in Notch signalling. The negative regulation of the ligand by receptor activation implied by this result is consistent with lateral inhibition models of Notch action in *Drosophila* (Heitzler and Simpson, 1991; Heitzler et al., 1996) and *C. elegans* (Wilkinson et al., 1994). It is also consistent with the fact that *Dll1* expression in the neural tube is restricted to individual, spatially separated cells (Henrique et al., 1995). On the contrary, *Notch1* is normally expressed in all or almost all cells in the same regions (Franco del Amo et al., 1992; Reaume et al., 1992). It is interesting to note, however, that *Dll1* expression in the presomitic mesoderm

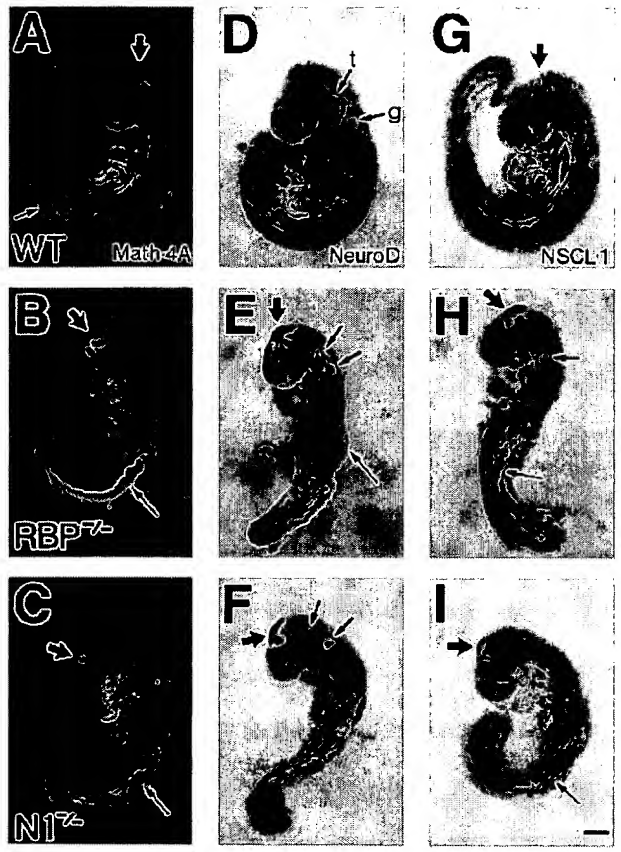

Fig. 6. Increased expression of early neuronal differentiation markers in *RBP-Jk* and *Notch1* mutants. E9.0 wild-type embryo (A,D,G); E9.0 *RBP-Jk* embryo (B,E,H); E9.0 *Notch1* embryo (C,F,I). (A) *Math-4A* expression in a wild-type embryo. Signal localizes to the ventral midbrain (arrow) and along the basal plate of the spinal cord (thin arrow). (B) *Math-4A* expression in a *RBP-Jk* mutant embryo. Signal is strongly up-regulated in the midbrain (arrow) and spinal cord (thin arrow). (C) *Math-4A* expression in a *Notch1* mutant embryo. Signal is up-regulated in the midbrain (arrow) and spinal cord (thin arrow). (D) *Neuro-D* expression in a E9.0 wild-type embryo. Signal is detected in the developing trigeminal (t) and geniculate (g) ganglia. (E) *Neuro-D* expression in a *RBP-Jk* embryo. Up-regulated signal is observed in the midbrain region (thick arrow) and in the developing trigeminal and geniculate ganglia (arrows). Expression in the midbrain overlaps with the region of up-regulated *Mash-1* expression. The dorsal neural tube also shows *neuro-D* signal (thin arrow). (F) *Neuro-D* expression in a *Notch1* embryo. Up-regulated expression is observed in the midbrain (thick arrow), trigeminal and geniculate ganglia (arrows). (G) *NSCL-1* expression in a wild-type embryo. Weak signal is observed in the midbrain region (arrow). (H)*NSCL-1* expression in a *RBP-Jk* mutant embryo. Note up-regulated expression in the midbrain (thick arrow), geniculate placode (small arrow) and in the neural tube (thin arrow). (I) *NSCL-1* expression in a *Notch1* mutant embryo. Up-regulation is observed in the midbrain (thick arrow) and neural tube (thin arrow) regions. Bar, 80 μm.

does not change in the mutants. Moreover, *Dll1* expression in the presomitic mesoderm of wild-type embryos is widespread and relatively homogeneous. This result suggests that the mechanism of action of Notch signalling in the presomitic mesoderm is not by lateral inhibition at the single cell level.

In *Drosophila*, a connection between the up-regulation of *E(spl)* and the downregulation of *Dl*, after activation of Notch signalling, is provided by genes of the *ac-sc* complex (Kunisch et al., 1994; Heitzler et al., 1996). The bHLH transcription factors encoded by *E(spl)* (Klaembt et al., 1987), negatively regulate the expression of the neural phenotype in the receiving cell (Fig. 7A, Heitzler et al., 1996). *Dl* expression is positively regulated by ac-sc, so that reduced ac-sc leads to downregulation of *Dl*, providing a feedback loop to link the expression of *Dl* and *N* (Fig. 7A). In the mouse, the functional equivalents of *ac-sc* are not clear. *Mash-1*, which is a homologue of *ac-sc*, is up-regulated in *RBP-Jk* and *Notch1* mutants, as predicted by the *Drosophila* model (Fig. 7A). However, *Mash1* cannot be the only regulator of *Dll1* expression, because it is not up-regulated throughout the expanded domain of *Dll1* expression in *RBP-Jk* and *Notch1* mutants. Mutational analysis of *Mash1* only revealed a role for the gene in the later differentiation of subsets of neural precursors in the PNS (Guillemot et al., 1993), consistent with the possible existence of additional genes overlapping in function with *Mash-1* in early neurogenesis. The recently described *neurogenin* may be such a candidate (Ma et al., 1996). There is no evidence on whether *Mash-1* or related genes directly regulate *Dll* expression in vertebrates. There is however, some evidence supporting a role for *Hes* genes in downregulating *Mash-1*, both in tissue culture experiments (Sasai et al., 1992) and from targeted mutagenesis in vivo (Ishibashi et al., 1995).

The mechanism by which the Notch signal is transduced to the nucleus is under intense investigation (see Introduction). In this study, we have used an affinity-purified polyclonal antiserum to examine the expression of RBPJK and determine if the subcellular localization of the protein changes during mouse embryogenesis. Western blot analysis indicated that RBPJK is specifically localized in the nucleus throughout development. Immunostainings revealed that RBPJK is widely expressed in the E8.5 mouse embryo and appears to be restricted to the nucleus (Fig. 2B, C). In addition, absence of the Notch1 receptor does not affect the subcellular localization of RBPJK in any region of the embryo, including the somites, where phenotypic effects of the *Notch1* mutation were observed. This indicates that the protein localizes stably in the nucleus, independently of the presence or absence of Notch1. These results would suggest that Notch signalling in the mouse embryo does not require major shifts in the subcellular localization of RBPJK and are consistent with the possibility that it is the movement of a proteolitically cleaved form of Notch to the nucleus that is critical in the signalling process (Jarriault et al., 1995; Tamura et al., 1995; Kopan et al., 1996). An additional possibility is that other Notch receptors in the absence of Notch1, would signal through RBPJK, thus affecting its subcellular localization.

A neurogenic phenotype in the mouse

A major issue in the study of vertebrate Notch signalling has been whether the neurogenic function that the pathway has in *Drosophila* is conserved in vertebrates. Overexpression of wild-type or dominant negative *Delta* constructs has provided support for such a role in primary neurogenesis in *Xenopus* (Chitnis et al., 1995). However, in mice, primary neurons do not form, and the role for Notch signalling in more general regulation on the transition from neural stem cell to committed

neuronal precursor has been unresolved. Loss-of-function mutations are essential tools to test whether the murine Notch pathway has a role in neurogenesis. Initially, the analysis of mutant embryos was inconclusive, since *Notch1* and *RBP-Jk* mutant embryos are developmentally retarded and begin to degenerate at about the time that the first neurons express their mature, differentiated phenotype (Swiatek et al., 1994; Conlon et al., 1995; Oka et al., 1995). The problem of early lethality has been circumvented in the present report through the analysis of markers of neuronal determination and early differentiation at stages before developmental arrest. The increased expression of *Dll-1, Mash-1, Math4A, neuroD* and *NSCL-1* strongly suggests that an excess of committed neuronal precursor cells are generated in the *Notch1* and *RBP-Jk* mutants at E9.0. This may well represent premature neuronal differentiation and a loss of stem cells in the nervous system. However, this cannot be assesed directly, since *both RBP-Jk* and *Notch1* mutants die shortly after E9.5. Thus, activation of the Notch pathway normally suppresses the formation of neurons, as it does in *Drosophila*.

The neural tube early in embryogenesis consists of a relatively homogeneous population of rapidly proliferating cells (Hartenstein, 1989, 1993; McConnell, 1981; Sechrist and Bronner-Fraser, 1991). Neurons are the major differentiated cell type generated by the early neural tube; glial cells arise only at later stages (Maier and Miller, 1995; McConnell, 1995). Thus, most neural tube cells may face a simple binary decision between remaining a neural tube cell or differentiating into a neuron. However, this process must be tightly regulated in time and space. Our results lead us to suggest that the Notch pathway regulates this decision in the mouse, by a feedback mechanism between differentiating neurons and the remaining neural stem cells.

A model for Notch-mediated regulation of murine neurogenesis

From our results and studies in other vertebrate systems in vivo and in vitro, it is possible to draw a tentative model for the role of Notch signalling in vertebrate neurogenesis that can be directly compared to the current *Drosophila* model (Fig. 7A). In this model, prospective neurons express the Notch ligand *Dll1* (Fig. 7B). Binding of Dll1 to Notch proteins on the adjacent cell, causes these cells to proteolytically process Notch (Jarriault et al., 1995; Kopan et al., 1996). The Notch cytoplasmic fragment translocates to the nucleus where it binds to RBPJK (Tamura et al., 1995; Hsieh et al., 1996). Notch/RBPJK complexes stimulate transcription of *Hes-5* by binding to the *Hes-5* promoter. Activation of RBPJK by Notch in the receiving neural tube cell, also leads to the repression of *Mash-1* and other genes involved in neuronal development, including the Notch ligand Dll1. We have shown that mutation of either the receptor Notch1, or its downstream effector RBPJK, leads to downregulation of *Hes-5* and concomitant upregulation of *Dll1* in the usual domain of expression of *Hes-5*. By analogy to *Drosophila*, we propose that Hes-5 does not repress *Dll1* directly, but acts indirectly via repression of *Mash-1* and other genes of the 'proneural' class (gene X: Neurogenin?, Fig. 7B), leading secondarily to repression of *Dll1*. *Mash1* cannot be the only regulator of *Dll1* expression, because it is not up-regulated throughout the expanded domain of *Dll1* expression in *RBP-Jk* and *Notch1* mutants. There is as yet no direct evidence for this part of the pathway, although the expression results are consistent with it. Since the cell that expresses Dll1 inhibits all the neighbors that it contacts from also expressing the ligand, Notch signal transduction does not occur in the prospective neuron. Thus, in the prospective neuron *Mash-1* and *neuroD* are highly expressed, and *Hes-5* is not. Mash-1 could in turn stimulate the expression of *Dll1*, completing the regulatory loop. Subsequently, the prospective neuron would commence its differentiation and would downregulate expression of *Dll1* or migrate away, allowing additional neuronal precursors to form. Although our data and the data of others support direct physical interaction or direct gene regulation from Dll1 to Notch to RBPJK to *Hes-5*, the existing data do not permit us to distinguish direct from indirect interactions in the other steps of our model.

The expression of all genes examined in this study was affected to a lesser extent by the *Notch1* mutation than by the *RBP-Jk* mutation, consistent with the weaker phenotype of *Notch1* mutant embryos (Swiatek et al., 1994; Conlon et al.,

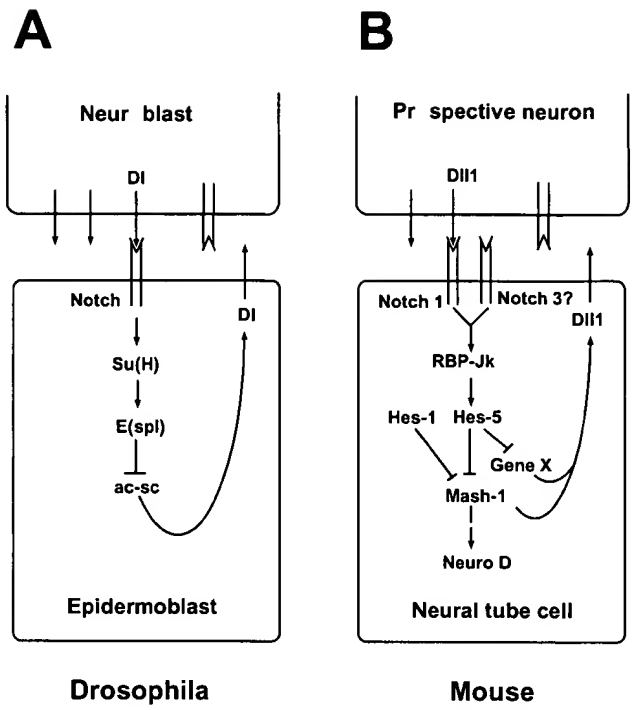

Fig. 7. Conservation of the Notch signalling pathway between *Drosophila* and the mouse. (A) Regulatory loop between Notch and Delta during lateral inhibition in *Drosophila* neurogenesis (modified from Heitzler et al., 1996). All competent cells express *Notch*, *Su(H)*, E(*spl*), *ac-sc* and *Delta*. After binding of Delta to Notch, the inhibitory signal is transduced to the nucleus via Su(H), activating *E(spl)* that represses *ac-sc*. *Delta* expression in the receiving cell probably depends on ac-sc activity. (B) Proposed regulatory loop between Notch1 and Dll1 during neurogenesis in the mouse. A prospective neuron expressing Delta activates Notch1 in a surrounding neural tube cell. The inhibitory signal is transduced to the nucleus via RBPJK that activates *Hes-5* expression, repressing *Mash-1* and probably another, as yet unidentified gene (gene X: Neurogenin?). *Dll1* expression in the receiving neural tube cell would depend on *Mash1* and gene X activity. The final result is the inhibition of neuronal differentiation in the receiving cell.

1995). We suggest that other *Notch* genes, such as *Notch3* (Lardelli et al., 1994), may have overlapping functions with *Notch1*, whereas *RBP-Jk* is required downstream of both genes. Thus, *RBP-Jk* appears to be a non-redundant element of the pathway, although recent analysis indicates the existence of a new *RBP-Jk*-related gene (T. Honjo, unpublished data).

Conclusion: *Drosophila* and mouse neurogenesis

The apparent conservation of function of Notch signaling in neurogenesis is surprising given the fundamental differences between insect and vertebrate neurogenesis. In *Drosophila*, the decision mediated by Notch is one between the epidermoblast and neuroblast lineages, both of which involve subsequent cell division. In the mouse and in other vertebrates, the neurogenic ectoderm is set aside from the surface ectoderm by an inductive interaction with mesoderm, which does not require the activity of the Notch pathway. It is the later decision between postmitotic neuron and proliferating neural epithelium that is regulated by Notch in vertebrates. Further genetic analysis combined with biochemical studies should shed light on how this important signalling pathway works in other aspects of normal development, and how its disruption can lead to tumorigenesis (Ellisen et al., 1991; Girard et al., 1996).

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Conservation of the expression and function of *apterous* orthologs in *Drosophila* and mammals

DIEGO E. RINCÓN-LIMAS*†, CHENG-HSIN LU*†, INMACULADA CANAL‡, MANUEL CALLEJA§, CONCEPCIÓN RODRÍGUEZ-ESTEBAN¶, JUAN CARLOS IZPISÚA-BELMONTE¶, AND JUAN BOTAS*‖

*Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030; ‡Departamento de Biología, Facultad de Ciencias-UAM, Madrid 28049, Spain; §Centro de Biología Molecular, CSIC-UAM, Madrid 28049, Spain; and ¶Gene Expression Laboratory, The Salk Institute, La Jolla, CA 92037

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ABSTRACT **The *Drosophila apterous* (*ap*) gene encodes a protein of the LIM-homeodomain family. Many transcription factors of this class have been conserved during evolution; however, the functional significance of their structural conservation is generally not known. *ap* is best known for its fundamental role as a dorsal selector gene required for patterning and growth of the wing, but it also has other important functions required for neuronal fasciculation, fertility, and normal viability. We isolated mouse (*mLhx2*) and human (*hLhx2*) *ap* orthologs, and we used transgenic animals and rescue assays to investigate the conservation of the Ap protein during evolution. We found that the human protein LHX2 is able to regulate correctly *ap* target genes in the fly, causes the same phenotypes as Ap when ectopically produced, and most importantly rescues *ap* mutant phenotypes as efficiently as the fly protein. In addition, we found striking similarities in the expression patterns of the *Drosophila* and murine genes. Both *mLhx2* and *ap* are expressed in the respective nerve cords, eyes, olfactory organs, brain, and limbs. These results demonstrate the conservation of Ap protein function across phyla and argue that aspects of its expression pattern have also been conserved from a common ancestor of insects and vertebrates.**

As DNA sequence data generated by the genome projects fill the databases, an increasing number of genes related by sequence are being identified in the human and model systems genomes. These sequence comparisons are expected to provide invaluable insight into the function of human genes (for example see ref. 1). In some cases, genes related by sequence are known to play the same or similar functions in distantly related organisms. Some well-known examples are the Hox genes involved in antero-posterior patterning (refs. 2–4 and references therein), the *dpp/bmp4, sog/chordin* genes involved in embryonic dorso-ventral patterning (reviewed in refs. 5, 6), the *Pax-6/eyeless* genes involved in eye determination (7), the *otd/otx* genes (8), and genes involved in certain signaling pathways (9, 10). In most cases, however, it is not known whether structurally related genes play the same roles in different organisms. To address the question of conservation of gene function, cross-species approaches that include comparisons of expression patterns and functional assays *in vivo* are required.

Here we use these approaches to investigate the conservation between *Drosophila apterous* (*ap*), a member of the LIM-homeobox gene family, and its mammalian orthologs. LIM-homeobox genes encode proteins containing two N-terminal zinc-finger-like motifs, referred to as LIM domains, in addition to a homeodomain. These genes have been found in very different organisms, including vertebrates and invertebrates, and are involved in tissue patterning as well as in cell fate determination and differentiation (reviewed in refs. 11–13). Some examples are: *Lhx1* required for the formation of the prechordal mesoderm (14), *Lhx3* required for the specification of pituitary cell lineages (15), *Isl1* required for the specification of motor neurons (16), and *Lmx1* required for limb dorso-ventral patterning (17–19). In *Caenorhabditis elegans*, *lin-11* and *mec-3* are essential for the development of vulval precursors and mechanosensory neurons, respectively (20, 21). In *Drosophila*, *islet* is required for axon pathfinding and neurotransmitter identity (22) and *arrowhead* for the development of certain imaginal cells (23).

ap was the first LIM-homeobox gene isolated in *Drosophila* (24, 25) and is best known for its crucial role as a dorsal selector gene required for dorso-ventral patterning and growth of the wing (26–28). However, mutations in *ap* cause a variety of mutant phenotypes illustrating other functions of *ap* during development. For instance, *ap* mutant embryos lack specific muscles (28) and show neuronal fasciculation defects (29). In addition, *ap* mutants die within few days after eclosion from the puparium (24), and they are deficient in juvenile hormone, which leads to nonvitellogenic ovaries and low female sexual receptivity (30).

Many LIM-homeobox genes have been conserved during evolution; however, the functional significance of their structural conservation is generally not known (reviewed in ref. 12). We have isolated mouse (*mLhx2*) and human (*hLhx2*) *ap* orthologs. We used ectopic expression and rescue assays to investigate the extent of the functional conservation between the *Drosophila* and human genes. In these *in vivo* assays, the human and fly proteins are interchangeable. In addition, we found striking similarities in the expression patterns of the *Drosophila* and murine genes. These results demonstrate the conservation of Ap protein function across phyla and argue that aspects of its expression pattern have been conserved also from a common ancestor of insects and vertebrates.

MATERIALS AND METHODS

cDNA Isolation. Two degenerate oligonucleotide pools were synthesized on the basis of two amino acid sequences conserved among Ap and other LIM-HD proteins. The sense primer [5′-GTI(G/T)TICAC(A/G)TI(A/G)AITG(T/C)TT(T/C)IIITG-3′] (I, inosine) was directed to the amino acid sequence VxHxxCFxC of the LIM2 domain. The antisense primer [5′-CGIII(A/G)TT(T/C)TG(A/G)AACCAIAC(T/C)TG-3′] corresponds to the amino acid sequence QVW-FQNxR present in the third helix of the homeodomain. Several cDNA fragments were amplified by using a mouse embryonic cDNA library. One of these fragments encoded an ORF with

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Abbreviations: *ap*, *apterous*.; VNC, ventral nerve cord; *fng*, *fringe*; *Ser*, *Serrate*; *vg*, *vestigial*; *wg*, *wingless*; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; UAS, upstream activation sequence.

Data Deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF124734 (*mLhx2*) and AF124735 (*hLhx2*)].

†These authors contributed equally to this work.

‖To whom reprint requests should be addressed at: Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza S942, Houston TX 77030. e-mail: jbotas@bcm.tmc.edu.

a high degree of similarity to the Ap homeodomain and was used to screen a λSH/*lox*-1 cDNA library prepared from E11 mouse embryos. The *mLhx2* cDNA (1.8 kb) was recovered from clone pSH340 and used as probe to screen a human brain λZAPII cDNA library (Stratagene). The *hLhx2* cDNA (2 kb) was recovered from clone p3B3A. Amino acid sequence comparisons were conducted by using the PILEUP program.

Generation of *Upstream Activation Sequence (UAS):ap* and *UAS:hLhx2* Lines. A 1.7-kb fragment containing the full *ap* ORF was cloned into the *Kpn*I site of pUAST (31). The 2 kb *hLhx2* cDNA was released from p3B3A and subcloned into the same vector. These constructs were introduced (32) into *yw; ap*^{UGO35}/CyO*wglacZ* (*wg, wingless)* flies. Several independent lines were generated in each case, all of which exhibit similar rescuing abilities (data not shown).

***Drosophila* Stocks.** The following stocks were used: *ap-GAL4*^{MD544} (33); 32B-GAL4 and *UAS:lacZ* (31); *UAS:tau-GFP* (34); *35UZ-1, fringe* (*fng*)-*lacZ* (35), and *vestigial* (*vg*) dorsal/ventral boundary enhancer-*lacZ* (36). *ptc*-GAL4 and *dpp*-GAL4 were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). *ap*^{rk568}, an enhancer detector line that expresses *lacZ* in the nuclei of *ap*-expressing cells, as well as the *ap* null allele *ap*^{UGO35}, were described previously (24). The *yw* strain was used for *in situ* RNA hybridization and immunodetection of Ap.

***Drosophila* Crosses.** The experiments shown use lines *UAS:apF29B* and *UAS:hLhx2F7A*, which contain the P element insertions in the X chromosome. For rescue experiments, *ap*^{UGO35}/CyO*wglacZ* females carrying either the *UAS:ap* or the *UAS:hLhx2* transgene were crossed to *ap-GAL4*^{MD544}/CyO*wglacZ* males. Females lacking the *UAS* trangenes were used as negative control. For ectopic assays, males from these UAS lines were mated to females carrying the 32B-GAL4, *ptc*-GAL4, and *dpp*-GAL4 drivers. The P[*35UZ-1*] (*fng-lacZ*), P[*vg-lacZ*], and P[*wg-lacZ*] insertions were introduced independently into the UAS lines and then crossed to the *dpp*-GAL4 driver to study the regulation of *ap*-downstream genes. The *ap* VNC-GAL4 driver, which expresses GAL4 from the 2-kb *ap*-ventral nerve cord (VNC) enhancer (D.E. R.-L. & J. B., unpublished data) was crossed to *UAS:tau-GFP* to label the *ap*-expressing neurons in the central nervous system.

Antibody and 5-Bromo-4-chloro-3-indolyl β-D-Galactoside (X-Gal) Stainings. Antibody staining of imaginal discs was performed as described (37). Mouse monoclonal anti-β-galactosidase antibodies (1:2,000; Promega), rat anti-Serrate serum (1:1,000; kindly provided by K. Irvine) and rat anti-Ap serum (37) were used. Ap immunodetection in adult head sections was conducted as outlined (38). For histochemical detection of β-galactosidase, wing discs and adult heads were stained with X-Gal (31) for 30 min to 2 h.

***In situ* RNA Hybridization.** Whole-mount *in situ* RNA hybridization in *Drosophila* embryos was carried out as outlined (39). Linearized *ap* cDNA was used as template in the digoxigenin-UTP RNA labeling kit (Boehringer Mannheim) to prepare sense and antisense probes. Whole-mount and sectional *in situ* RNA hybridization in mouse embryos was conducted as described (40). Digoxigenin- and ^{35}S-labeled *mLhx2* riboprobes were prepared from clone pSH340. Sections hybridized with sense probes did not reveal any specific signal (data not shown).

RESULTS

Cloning and Sequence of *mLhx2* and *hLhx2*, the Murine and Human Orthologs of *apterous*. The murine ortholog of *ap* (*mLhx2*) was isolated by using PCR and degenerate primers corresponding to the homeobox and LIM2 domain of the *ap* cDNA. The PCR product was then used to obtain a full-length cDNA from a mouse embryonic library. The human *ap* ortholog (*hLhx2*) was isolated by using the mouse cDNA to screen a human brain library (see *Materials and Methods*).

Fig. 1*A* shows the amino acid sequence comparison of the fly, mouse, and human proteins. MLHX2 and HLHX2 differ only in four amino acids except for an extended amino terminus of the mouse protein. The fly and mouse/human proteins show three major domains of sequence similarity that correspond to the LIM domain 1 (57% identity), LIM domain 2 (56% identity), and homeodomain (93% identity). However, conserved amino acids are found also outside these domains (Fig. 1*A*). Fig. 1*B* shows that the percent identities between Ap and its mammalian orthologs are clearly higher than between Ap and other LIM-homeodomain proteins.

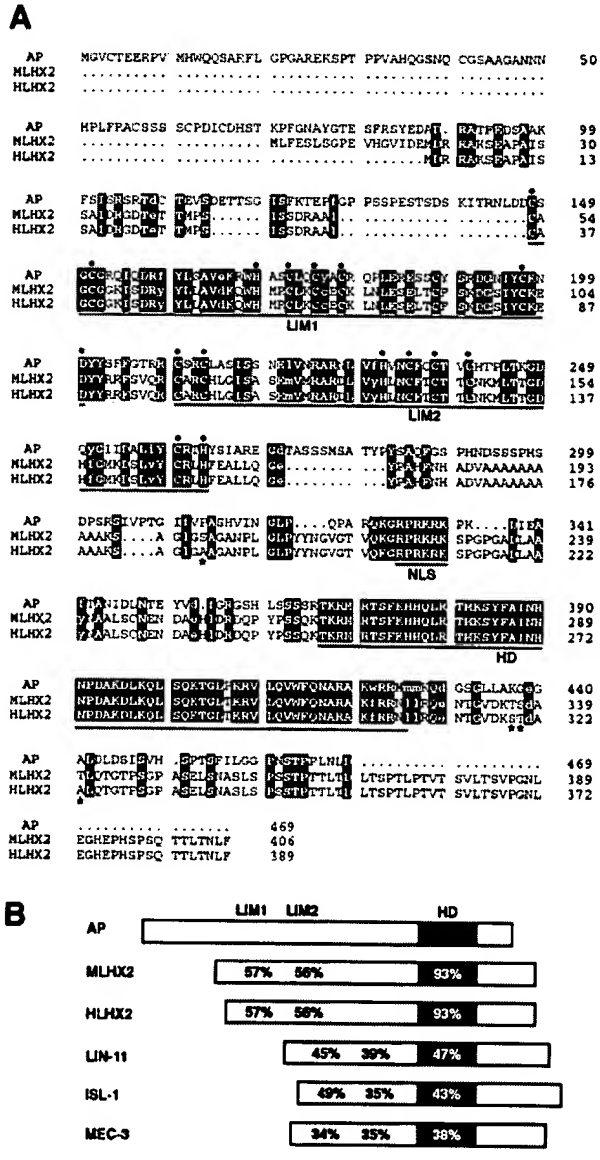

FIG. 1. Amino acid sequence comparison of *Drosophila* Ap and its mouse (MLHX2) and human (HLHX2) orthologs. (*A*) Sequence alignment. Identical amino acids between the three proteins are displayed in reverse type with capital letters, whereas conservative substitions are displayed in lower case letters. Asterisks indicate the only four different residues between the mouse and human proteins in the overlapping region. The two tandem LIM domains, the putative nuclear localization signal (NLS), and the homeodomain (HD) are underlined. The consensus residues of the LIM domains are highlighted with black circles. Gaps denoted by dots have been inserted to maximize sequence alignment. (*B*) Domain comparisons between *Drosophila* Ap, its mammalian orthologs, and other LIM-homeodomain proteins. These are: ISL-1 from rat (60), LIN-11 and MEC-3 from *C. elegans* (20, 21). Percentage of amino acid sequence identity is indicated within the LIM domains and homeodomain.

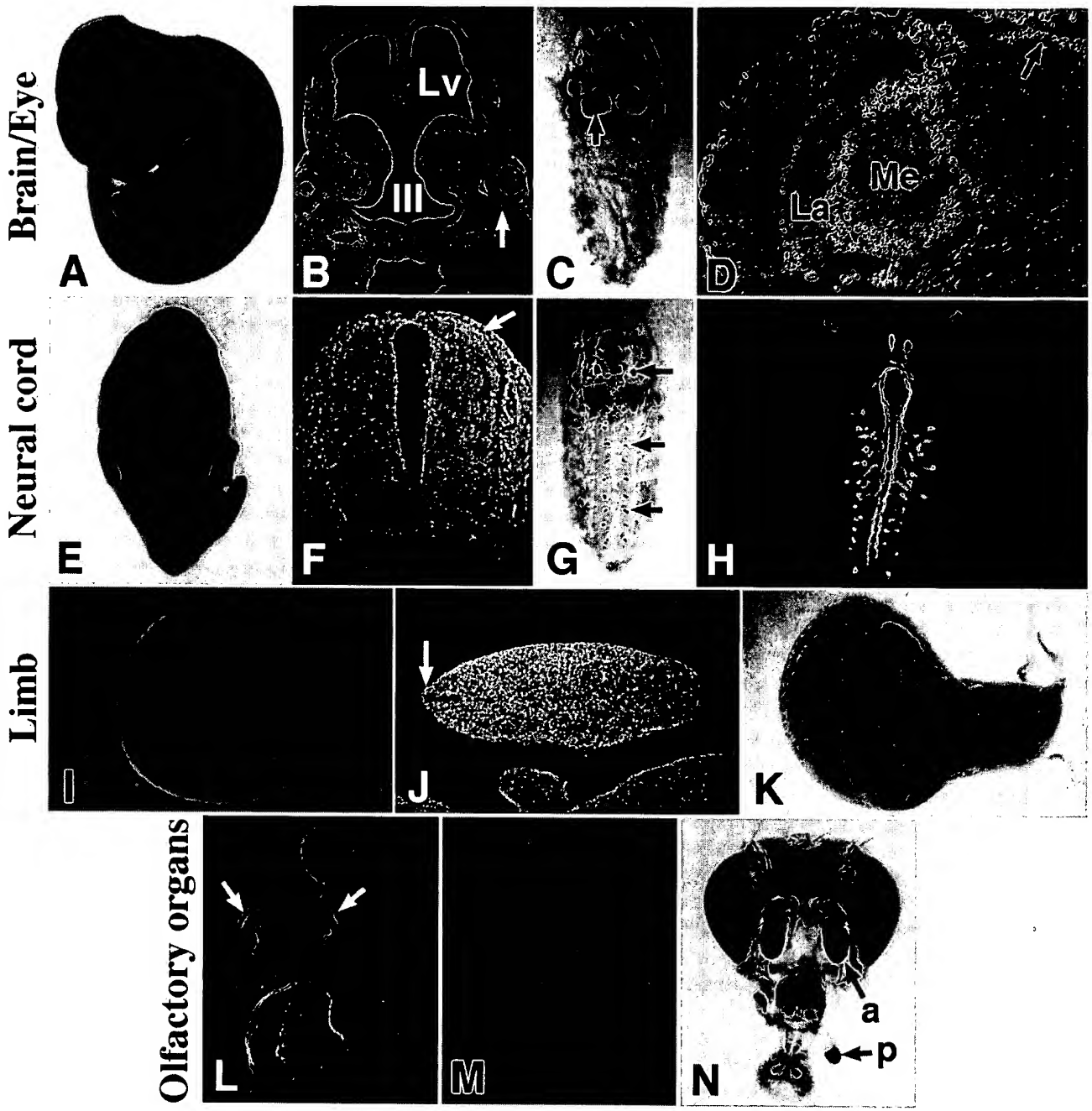

FIG. 2. Comparison of *ap* and *mLhx2* expression patterns. (*A*) *mLhx2* expression in the forebrain and limbs of an E11.5 mouse embryo. (*B*) At this stage, *mLhx2* is expressed in the walls of the lateral ventricles (Lv) and third ventricle (III) of the brain. In the eyes, *mLhx2* is expressed in the future nervous layer of the retina (arrow) and in the optic stalk (not shown). (*C*) *ap* expression in the brain hemispheres (arrow) of a stage 15 fly embryo. (*D*) In the adult fly, Ap is immunodetected in the lamina (La) and medulla (Me) of the optic lobe and in the central brain (arrow). (*E* and *F*) At E11.5, *mLhx2* is expressed along the neural tube (*E*) in a group of dorsal commissural interneurons (arrow in *F*). (*G*) *ap* expression in the VNC (arrows) of a stage 15 fly embryo. Out of focus, expression is also evident in the brain hemispheres and muscles of the body wall and pharynx. (*H*) *Drosophila* larval central nervous system showing expression of a *UAS:tau-GFP* responder driven by the *ap*-VNC enhancer. Note the axonal projections of *ap*-expressing interneurons along ascending longitudinal tracts. (*I* and *J*) *mLhx2* expression in E11.5 mouse limbs. Label is detected in the mesenchyme, in a region roughly corresponding to the progress zone (*I*). In cross-sections, *mLhx2* is observed in both dorsal (up) and ventral (down) regions of the limb and is excluded from the apical ectodermal ridge (arrow in *J*). (*K*) Ap immunodetection in the dorsal compartment of a *Drosophila* wing imaginal disc. (*L*) Section from an E11.5 mouse embryo showing *mLhx2* expression in the olfactory epithelium surrounding the nasal pits (arrows). (*M*) *ap* expression in the center of a *Drosophila* antennal disc. (*N*) X-Gal stain of a *Drosophila* adult head carrying the enhancer detector ap^{rk568}, which expresses *lacZ* in an *ap*-like fashion. Note *lacZ* expression in the fly olfactory organs: the antenna (a) and the palpus (p).

The MLHX2 protein is 90% identical to a rat protein known as rLH2 (41), probably the rat ortholog of *mLhx2*. Mouse genes related to the *mLhx2* gene described here have been reported elsewhere (41–43), but lack of sequence data prevents their comparison (see *Discussion*). The human Ap protein (HLHX2) is 92% identical to hLH2, a protein aberrantly expressed in chronic myelogenous leukemia (44). We mapped *hLhx2* to chromosomal region 9q33–34.1 by fluorescent *in situ* hybridization (data not shown), the same chromosomal region where *hLH2* maps (44).

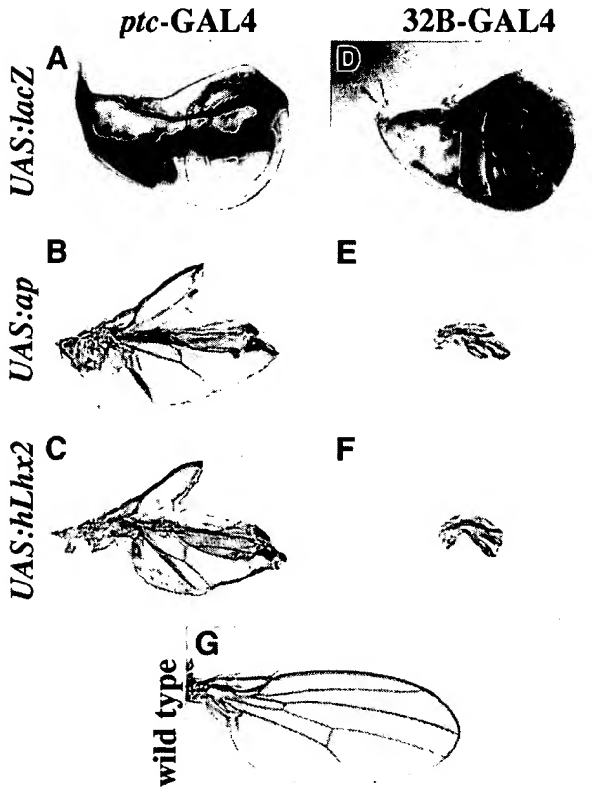

FIG. 3. Ectopic expression of *ap* and *hLhx2* in wing imaginal discs produce similar wing phenotypes. (*A* and *D*) *UAS:lacZ* expression from the *ptc*-GAL4 (*A*) and 32B-GAL4 (*D*) drivers. (*B* and *C*) Wing phenotypes caused by ectopic expression of *ap* (*B*) and *hLhx2* (*C*) by using the *ptc*-GAL4 driver. (*E* and *F*) Wing phenotypes caused by ectopic expression of *ap* (*E*) and *hLhx2* (*F*) by using the 32B-GAL4 driver. (*G*) Wild-type wing.

Similarities in the Expression of *apterous* and *mLhx2*. Expression of *mLhx2* was investigated by *in situ* hybridization to whole-mounted and sectioned E9.5–12.5 embryos. *mLhx2* expression was detected in the brain (Fig. 2 *A* and *B*), in the eyes (Fig. 2*B*), olfactory epithelium (Fig. 2*L*), and neural tube (Fig. 2 *E* and *F*). These patterns are reminiscent of *ap* expression in the embryonic and adult brain (Fig. 2 *C* and *D*), optic lobe (Fig. 2*D*), antenna, and maxillary palpus (the fly olfactory organs, Fig. 2 *M* and *N*), and VNC (Fig. 2*G*). The cells that express *ap* in the *Drosophila* VNC are interneurons, as revealed by driving expression of the *tau-GFP* reporter gene from the *ap* VNC enhancer (Fig. 2*H*); see also ref. 29. Thus we investigated the identity of the cells expressing *mLhx2* in the mouse neural tube. Fig. 2*F* shows a section through the neural tube; *mLhx2* label is detected in a dorso-lateral domain where dorsal commissural neurons are located. Interestingly, these are a subset of interneurons that, like *Drosophila ap* interneurons, send axons along longitudinal ascending tracts (45, 46). Other regions of *mLhx2* expression include the liver, the infundibulum of the pituitary, and a small region of the branchial arches in E9.5 but not older embryos (data not shown).

In addition, *mLhx2* expression was detected in the limb buds, specifically in the mesenchyme of the progress zone (Fig. 2 *A* and *I*) and excluded from the apical ectodermal ridge (Fig. 2*J*). Sections through the limb buds shows that *mLhx2* is expressed both dorsally and ventrally (Fig. 2*J*) in contrast to the dorsal-specific expression of *Drosophila ap* (Fig. 2*K*). Also unlike *ap*, we did not detect *mLhx2* expression in the somatic mesoderm.

***hLhx2* Correctly Regulates *apterous* Target Genes in *Drosophila* and Mimics *apterous* in Ectopic Expression Assays.** The conservation of the Ap amino acid sequence and expression patterns from *Drosophila* to mammals prompted us to investigate the possible conservation of its functions using *in vivo* assays. The yeast GAL4/UAS system (31) was used to drive expression of a *hLhx2* transgene in flies.

First we compared the phenotypic consequences of ectopic expression of *hLhx2* and *ap*. Fig. 3 *A* and *D* show the *lacZ* expression pattern in the wing imaginal disc from two GAL4 drivers used in these experiments. Fig. 3 *B* and *C* (*patched*-GAL4 driver) and Fig. 3 *E* and *F* (32B-GAL4 driver) show that the severe wing mutant phenotypes produced by ectopic expression of *ap* or *hLhx2* are virtually indistinguishable. For comparison Fig. 3*G* shows a wild-type wing.

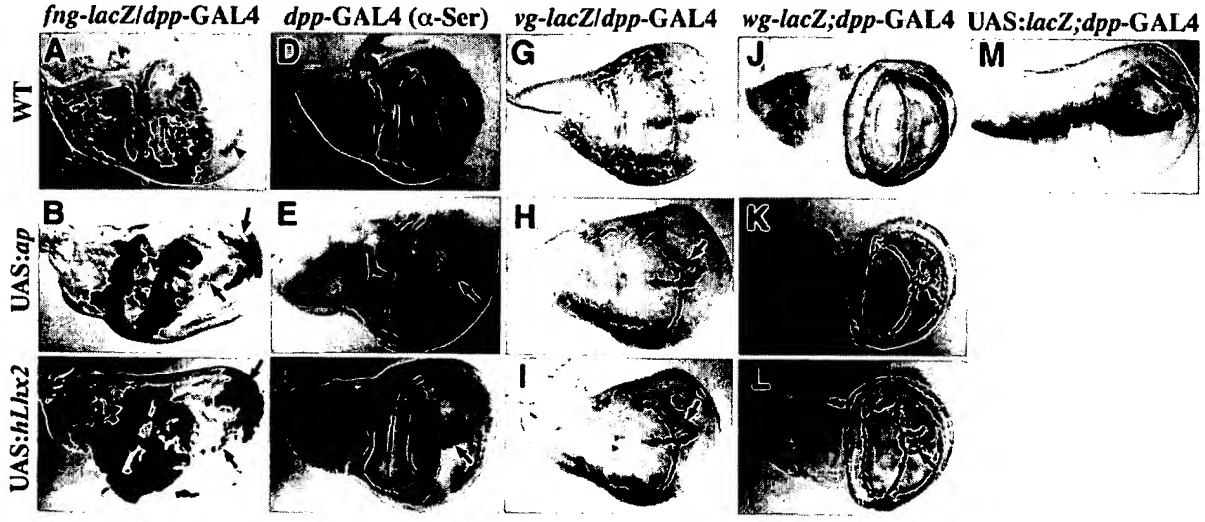

FIG. 4. hLhx2 correctly regulates *ap* target genes in *Drosophila* wing imaginal discs. Panels show β-galactosidase or Ser immunodetections following ectopic *ap* or *hLhx2* expression in third instar larvae wing discs. The wild-type pattern of the *fng-lacZ* (*A*), *Ser* (*D*), *vg-lacZ* (*G*), and *wg-lacZ* (*J*) markers are depicted at the top. The *dpp*-GAL4 driver (*M*) was used to direct expression of the indicated UAS transgenes along the antero-posterior axis. Note that in all cases wing discs coexpressing *dpp*-GAL4 and either *UAS:ap* (*B, E, H, K*) or *UAS:hLhx2* (*C, F, I, L*) exhibit ectopic activation of the molecular markers within the ventral compartment. Arrowhead indicates the wild-type expression of *fng* in the ventral compartment. Arrows point to the sites of ectopic expression.

In addition, we investigated whether *hLhx2* could regulate genes that are directly or indirectly under *ap* control, such as *fng*, *Serrate* (*Ser*), *vg*, and *wg* (35, 47). Using the *dpp-GAL4* driver (Fig. 4*M*), we expressed *UAS:hLhx2* and *UAS:ap* within the wing ventral compartment along the antero-posterior compartment boundary. Fig. 4*A* shows that *fng* expression in the wild-type wing disc is almost completely restricted to the dorsal compartment. Fig. 4 *B* and *C* show that *hLhx2*, like *ap*, activates *fng* expression in the ventral compartment. Fig. 4*D* shows the *Ser* wild-type expression pattern, which is also restricted to the dorsal compartment of the disc. Ectopic expression of *ap* and *hLhx2* produced similar activation of Ser expression along the antero-posterior boundary (Fig. 4 *E* and *F*). Fig. 4 *G* and *J* show the wild-type expression patterns of *vg* and *wg* along the dorsal-ventral compartment boundary, respectively. On *ap* ectopic expression, *vg* and *wg* are ectopically activated as two parallel stripes within the ventral compartment (Fig. 4 *H* and *K*). These regulatory interactions are mimicked by *hLhx2* ectopic expression (Fig. 4 *I* and *L*). As expected, the adult wings resulting from these crosses exhibit an ectopic wing margin along the ventral compartment (data not shown).

Rescue of *Drosophila apterous* Mutant Phenotypes by a *hLhx2* Transgene. To test further the conservation of Ap protein functions from flies to humans, we investigated the ability of HLHX2 to substitute for Ap functions during *Drosophila* development.

We took advantage of a GAL4 enhancer detector inserted in the *ap* locus (33). Insertion of the GAL4 P-element in *ap* causes GAL4 to be expressed like *ap* (Fig. 5*A* and data not shown). This insertion also results in a strong *ap* mutation leading to the lack of wings and halteres, as well as a mutant notum that lacks the scutellum and many of the bristles (Fig. 5*B*). In addition, these mutants also show the sterility and precocious death phenotypes associated with strong *ap* mutations (they have a life span of 1–3 days after eclosion from the puparium; data not shown). *ap* mutant flies carrying the *ap-GAL4*^{MD544} allele and the *UAS:hLhx2* transgene show rescue of the *ap* wing, haltere, scutellum, and bristle mutant phenotypes (Fig. 5*D*). The sterility and precocious death phenotypes are also rescued (data not shown). We find that the fly (*UAS:ap*) and human (*UAS:hLhx2*) transgenes are equally able to rescue these phenotypes (see Fig. 5 *C* and *D*). The only difference that we detected between the two rescue transgenes is that flies that carry *UAS:hLhx2* frequently develop one to two extra bristles in the scutellum, a phenotype not observed with *UAS:ap*.

DISCUSSION

Many proteins show a remarkable degree of amino acid sequence conservation between distantly related species.

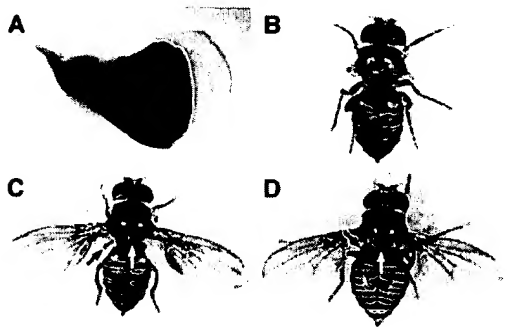

FIG. 5. *hLhx2* rescues the wing phenotype of *ap* mutants. (*A*) Wing imaginal disc expressing *UAS:lacZ* from the *ap-GAL4*^{MD544} driver. This GAL4 P-element insertion in *ap* inactivates the gene and recapitulates its expression pattern. (*B*) *ap-GAL4*^{MD544}/*ap*^{UGO35} mutant fly. Note the lack of wings, halteres, and the scutellum region of the notum. (*C* and *D*) *ap-GAL4*^{MD544}/*ap*^{UGO35} mutant flies carrying the *UAS:ap* and *UAS:hLhx2* transgenes, respectively. Note that, in both cases, the wing, notum (white arrow), and haltere (black arrow) phenotypes are rescued.

However, apparent structural conservation does not necessarily imply conservation of function *in vivo*. We have used transgenic animals and rescue assays to investigate the functional conservation of the Ap LIM-homeodomain protein during evolution. We found that the human protein HLHX2 is able to correctly regulate *ap* target genes in the fly, causes the same phenotypes as Ap when ectopically produced, and most importantly rescues *ap* mutant phenotypes as efficiently as the fly protein. These observations provide compelling evidence for the functional conservation of the Ap protein.

Other putative *ap* orthologs have been identified in vertebrates and invertebrates. In *C. elegans*, a LIM-homeobox gene closely related to *ap* is expressed in a specific interneuron required to mediate thermoregulation (48). In the crustacean *Artemia franciscana*, a putative *ap* ortholog is expressed in gill appendages (49). In vertebrate genomes, it appears that more than one *ap*-related gene is present. Paralogous genes closely related to *ap* have been reported in the chicken (50) and in the zebrafish (H. Okamoto, personal communication). Mouse genes related to the *mLhx2* gene described here have been reported elsewhere (41–43). However, lack of reported DNA sequence and expression pattern data prevents their comparison. Thus we do not know whether these genes are the same as the *mLhx2* gene described here or its paralogs.

Analysis of the expression pattern of the mouse *ap* ortholog described here (*mLhx2*) shows that it is expressed in many organs and tissues that are analogous or homologous to the organs and tissues where *Drosophila ap* is expressed. These include the eye, olfactory organs, limbs, brain, and neural tube. Particularly interesting are the expression patterns of the fly and mouse genes in the respective appendages (wing imaginal discs and limbs) and nerve cords. What is the significance of *ap* and *mLhx2* similarities in their expression patterns? These similarities suggest that *ap* and *mLhx2* may play the same or similar roles in flies and mice during differentiation of the respective limbs, brains, nerve cords, eyes, and olfactory organs.

ap expression in the *Drosophila* wing imaginal disc is required for specifying dorsal vs. ventral identity and for growth of the appendage (26–28, 47). These two distinct functions carried out by *ap* are separable. *ap* mutant flies in which the *fringe* gene is driven by the *ap-GAL4* driver described here have normal-size wings that are double ventral (Jose de Celis, personal communication). As discussed below, these two functions of *Drosophila ap* are carried out in vertebrates by two different genes. In mice, *mLhx2* does not appear to be involved in dorso-ventral specification of the limb because it is expressed on dorsal and ventral sides (Fig. 2*J*). In chicks, an *ap/mLhx2* ortholog also shows dorsal and ventral limb expression (51). It is a different member of the LIM-homeobox family, *Lmx1*, the gene that specifies dorsal vs. ventral identity in vertebrates. Lack of *Lmx1* function results in normal-size limbs that are double ventral (17–19).

Mice deficient for one of the *ap* orthologs have been generated, demonstrating a requirement of this gene in development of the eye, cerebral cortex, and efficient definitive erythropoiesis. These mutant mice do not show any limb phenotype, probably because of functional redundancy with other mouse *ap* paralogs (51, 52). However, a dominant negative form of a chicken *ap* ortholog results in arrested limb outgrowth during embryogenesis (51). These results suggest a conserved function of *ap* required for appendage growth in *Drosophila* and vertebrates.

In the *Drosophila* VNC, *ap* is expressed in a small number of cells per hemisegment. The activation of the *tau-GFP* reporter gene by the *ap-GAL4*^{MD544} driver allowed us to visualize the projections of these interneurons. Interestingly, *in situ* hybridization on mouse neural tube sections shows that the cells expressing *mLhx2* precisely colocalize with a subset of interneurons that, like *Drosophila ap* interneurons, project along ascending longitudinal tracts to anterior segments

and/or to the brain. *Drosophila ap* mutants show neuronal pathfinding defects (29); thus these observations suggest a conserved role for *ap* in interneuron identity or pathfinding.

There is still considerable controversy on the homology between the nerve cord of protostomes and deuterostomes (53–55). Although the nerve cord of deuterostomes is located dorsally instead of ventrally in protostomes, recent molecular data support the hypothesis that they are homologous (reviewed in ref. 8). The difference is explained as a consequence of an inversion of the dorso-ventral body axis between arthropods and chordates (5, 6). In addition, limbs, olfactory organs, and eyes have been classically considered to be analogous between arthropods and chordates, but see refs. 7 and 56 for novel views in the cases of eyes and limbs, respectively. Thus, according to orthodox views, it would have to be argued that *ap* functions were recruited independently at more than one time during evolution for the development of these analogous organs where they may carry out similar functions. Whatever the evolutionary relationship between fly and mouse organs might be, the similarities in *ap* expression support the idea (57) of a common set of functionally related genes involved in the development of the respective organ. This group of genes is known as a syntagma. Thus functionally related organs or tissues in flies and mice would have similar apogenomes (the combination of active genes within a given cell or tissue; ref. 57).

The similarities between the expression patterns of *ap* and *mLhx2* leave some intriguing questions open for future investigations: What deep level of homology underlies their expression pattern similarities (58, 59)? Have *ap* regulatory elements been conserved between flies and mammals?

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